

Winning flexibility for research

The largest of the British research councils has produced a corporate plan that dodges all the hard questions. Here is a question that it cannot avoid much longer.

THE variety of names under which British organizations now canvass their hopes and aspirations has become bewildering. In the old days, before 1980, it was by no means unusual that research organizations such as the five British research councils would put out documents called "plans" or, a little more pretentiously, "strategies", from which interested readers might tell what might lie ahead. More recently, it has been the custom for the research councils to prepare each year a document called a "forward look" intended to accompany and sustain their annual requests for government funds. But now, in deference to the British government's conviction that research should be a branch of business, "corporate plans" are all the rage. The Agricultural and Food Research Council was the first in the field nearly a year ago. Now it has fallen to the Science and Engineering Research Council (SERC) to publish (see p.589) a document with such a title, but which is otherwise such a pale imitation of what a commercial corporation would put out under such a heading that there is a danger that the new currency will be debased even before its meaning has been generally understood.

SERC is worried about money, and for a good reason, as it has been for the past several years. The document published this week has its origins in the sense of panic that overtook the council in the autumn of 1984, when it was decided that nothing less than a radical reappraisal of the council's activities would suffice to make ends meet. It had already then been decided that British membership of the European high-energy physics enterprise (CERN) should be reconsidered. SERC off its own bat resolved to decide whether two home-based research observatories were needed to support activities at the overseas observatories, those with real telescopes. And there was to be a reappraisal of the fields of science in which the council would concentrate its future efforts. By all accounts, the steam went out of the enterprise when it was found that the budget for the current year would not be as meagre as at first feared, partly because of a share of the extra £15 million quarried by Sir Keith Joseph, the Secretary of State for Education and Science, from thin air, but at the expense of his political standing with his colleagues. Then, in the summer, came a new chairman and no fewer than seven new members of the council, no more willing to be bound by their predecessors' decisions (and prevarications) than would be a commercial board. The result is a document that still pleads the need for radical reappraisal, which nevertheless still lies somewhere in the future.

Because reappraisal is altogether too important for the health and welfare of the British research community to be left indefinitely on the shelf, and since SERC's problems are not in essence different from those of the other research councils operating in support of basic science in Britain, the new council should face up to the problems it and its predecessors have endlessly defined. The difficulty is that SERC has lost flexibility over the years by the growth of its apparently unavoidable commitments. The commitment to CERN, £35.5 million last financial year, which fluctuates with the value of sterling, is the most conspicuous of these. The two British-based observatories are another constraint on flexibility, and their future may be clearer when the committee on the subject under Sir John Kingman produces its report next year. But the end result is that, out of a total budget of £291 million in 1984-85, SERC was

able to spend only £79 million on research grants and £46 million on research fellowships of various kinds. Where has the rest of the budget gone? The conspicuous commitments are not the only restraints on the council's flexibility. The second largest item in SERC's budget, after research grants, is the cost of the establishment known as the Rutherford Appleton Laboratory (RAL), on the site of the now defunct NIMROD proton accelerator, where some 1,400 professional people provide support services for research in the physical sciences undertaken by the academic community. Should not a truly radical reappraisal consider whether such a large establishment, ostensibly for servicing grant spending which is nearly identical in amount, should continue to exist in its present form?

None of this should be read as a complaint against the laboratory or its staff. By demonstration over the past several years, they have done a splendid job in a variety of important fields. The laboratory is the chief centre in Britain for the development of instruments to be flown in Earth satellites in the pursuit of basic research. It is a computing centre of great value to the British academic community and also a source of innovation in the field. In its own right, the laboratory has an important programme of ionosphere and atmosphere research, a legacy of the merging of the old Appleton laboratory with the old Rutherford laboratory a decade ago. And RAL continues to mastermind some parts of nuclear physics, managing the spallation source that is now the chief source of academic neutrons in Britain. All this, and much else, has been done well. But SERC should ask itself whether such an establishment is, in present circumstances, more of a hindrance than a help.

The arguments for a change at RAL are powerful. As a laboratory whose chief function is to service academic research for the whole of a national research community, RAL is distinguished and probably also unique; nowhere else has such an all-purpose establishment been found necessary or thought wise. The most obvious difficulty is that a commitment on this scale to skilled people, however flexible they may be individually, cramps the council's strategic flexibility. Moreover, it is no longer likely that the work now sited at RAL is all of a kind that must be done centrally because "size and complexity" would overwhelm university sites at which it might be placed. And while SERC's corporate plan boasts modestly of some contract work for industry, when universities are as hungry as they are at present, it is much more likely that technology would be quickly transferred from where it is created if it had sprung from university laboratories, not centralized institutions.

Reading between the lines of the corporate plan, the council plans to be more timorous. There is talk of an agreement with the unions that 10 per cent of the staff at RAL might be on short-term contracts (but the quota has not yet been taken up). What SERC should be planning is a scheme for devolving as much as possible of the work that it does at RAL to universities. Just now, there is a further possibility that some part of RAL might be spun off to the new space centre on which the British government has set its heart. The dangers of such a move are clear in people's minds, but the advantages of the flexibility that could result are greater. The real danger, for SERC, is that it cannot convincingly threaten CERN and its observatories with the knife if the largest of its establishments remains untouched. □



Holding to tenure

The British government and British academics are both misguided on the tenure system.

THE howls of protest that might have been expected after last week's announcement that the British government is to fulfill its threat to abolish academic tenure have been surprisingly muted. Is it perhaps that academics know that it will not be them, or most of them, who will be affected, but their successors? But although the issue is for the time being relatively silent, there is every prospect of trouble when the bill that Sir Keith Joseph promised he would be introducing to the House of Commons later in the parliamentary session begins its passage. For the government's proposals will engender legislation of the worst kind, schemes for putting wrongs (which there are) to rights which are engendered not by a sober consideration of what needs to be done, but by animus. The best outcome would be that one or other of the houses of parliament should throw out what the government proposes, substituting legislation that would give it what it has a right to ask for — and giving academic institutions their just needs at the same time.

Sir Keith Joseph first advertised his plans early last summer. The problem he intends to tackle is the way in which many (but not all) contracts of employment between universities and their academics include the provision that people will have the right to stay at work until their normal retirement age unless previously dismissed for misconduct defined by a narrowly drawn list of misdemeanours. Because the constitution of British universities is defined by means of separate royal charters, legally untouchable by parliament, unless those governed by the charters (which means academics) choose to amend them, parliament and thus the government can act only indirectly, by persuading the Privy Council (the monarch's secretariat) to appoint commissioners with powers to amend the charters one by one. It is a fair guess that this decade will be out before the job is done, which is one reason why an ageing academic workforce may not be too alarmed. But the amendment that the minister now proposes has the further defect that it will apply only to some academics, those newly appointed or those who have the good fortune to be promoted from one grade in the hierarchy to another. So, for a transitional period that will last until the year 2025 or thereabouts, there will be two kinds of British academics — those with tenure and those who do not have it. Tidy government needs something better.

What the government has consistently failed to acknowledge is that academic tenure has a purpose, that of making sure that individual academics are not victimized for unorthodox opinions, a pitfall into which the most imaginative and ultimately the most valuable scholars are prone to fall. Nothing in what the government now proposes will change that state of affairs. What will happen is merely that younger academics in pursuit of unorthodox lines of enquiry will more easily be shown the door by conservative heads of department. Most senior academics, of course, will be too sensible for that, but that is not much of a safeguard against the consequences of the tetchy intellectual enmity that so often blows up in university atmospheres. In this respect, tenure is a necessary and valuable safeguard against intellectual injustice. It is shabby that the British government has let its resentment of the idea that academics "have a job for life" cloud its judgement about the need for a measure of academic freedom. Animus has got the better of it.

But none of this implies that tenure should be inviolable. That is the point at which academics are often wrong. If universities are, or wish to be, self-governing institutions, they must also be free to change course from time to time. If, for example, there should be no students wishing to read Sanskrit at the university at which it is best taught, the university must at least be free to decide whether the department concerned, and those who teach in it, should be dispensed with. Naturally, it would be again be shabby if a university, having seen the demand for Sanskrit fall to nothing, should be able to write out dismissal notices on the

spot, without further consultation. No academic institution seeking respect elsewhere could behave in such a cavalier fashion and then hold its head up (or attract other academics). Even in a free market, scholarship is worth paying for. To ensure that decisions such as these are made in a seemly fashion, two considerations must be satisfied. Universities must be well governed domestically, so that even the victims of hard decisions may feel they have participated in them. And universities must have enough autonomy, financially as well as legally, for good internal government to carry conviction.

How is that to be accomplished? This is where the British government is especially vulnerable to rational complaint. For the past five years, universities have been forced to live financially from hand to mouth. Even at this stage in the current academic year, British universities do not know what subsidy they will be given by the government in the year beginning eight months from now. They will not know for sure until the spring. How, in such circumstances, can universities pretend to themselves, and to those who work for them, that they are self-governing and autonomous? And even if the government is only playing cat-and-mouse with its universities, hoping to keep them hopping with uncertainty, how can it be sure that some of its assets will not give way under the strain? If the government had been more moderate on this contentious issue, it would probably have been able to win what it wants without the fuss now in prospect. What it needs is not a law but a deal, one in which the universities would trade a reasonable self-determined restraint on tenure for a measure of financial security of the kind the government has perversely chosen to deny them. If the government had followed that path, it might even have found falling into its lap the prize it has not yet dared to ask for — the abolition of the doctrine that academics should all be paid the same salaries (at the same age), however successful (or otherwise) the institutions at which they work. □

Poor solstice

Nature's annual weekly break coincides with the break in the calendar, on this occasion by design.

THE New Year festival is of course discriminatory. Its psychological significance is that of the winter solstice; *reculer pour mieux sauter* is the motto, which discriminates against those living in the Southern Hemisphere and even in the tropics. In due course, it will be acknowledged that an equinoctial break (but which?) would be more equitable. Meanwhile, the old northern conventions persist, even to gloomy Nordic reflection on the past and speculation about what lies ahead.

Here is how that train of thought might go. In retrospect, 1985 has been a teasing year. One proof of that is the Geneva summit, which happened; the let-down is that nothing much happened when it did. The US space shuttle has gone up and returned with great regularity (Ariane less reliably), but the seemingly quite recent excitement about journeys within the Solar System has been overshadowed by evidence of the physical weakness of people's hearts and minds.

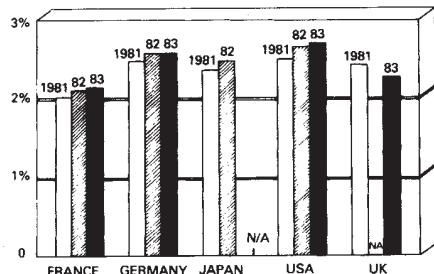
Science in the year past was a fine-textured business. Umpteen more nucleotide sequences cram the databanks, but there are only a couple of extra biogenetic chemicals on the market, and just a handful of gene probes that might spot congenital disease in advance. Most people have learned that, with computers, the software is more important than the hardware — and have taken to complaining about the former. No new fundamental particles have been found (the *W* and *Z* belong to 1984), and fundamentalism is out of fashion, in any case.

So will 1986 have a more exciting structure? Predictions being impossible, extrapolations must do instead. The spacecraft Giotto will get Halley's comet (being almost there), the US Congress will be petrified by the prospect of next November's elections, there will be a lot more nucleotide sequences and people will be rightly alarmed by the spread of AIDS, and the consequences thereof. More of that next year. □

UK research councils

Science council thinks ahead

BRITISH academic astronomers are seeking to persuade the Science and Engineering Research Council (SERC) to transfer the staffs of the two UK observatories to university research centres. So much has emerged in the past week, in advance of the publication of the SERC "corporate plan", published earlier this week. The plan says that one of the many questions



Expenditure on R&D as percentage of GDP 1981-83.

still unresolved about its future is that of the two observatories, the Royal Greenwich Observatory at Herstmonceux in Sussex and the Royal Observatory, Edinburgh, whose present functions are chiefly those of providing ground support for British telescopes at La Palma in the Canary Islands and on Hawaii.

Astronomers at the University of Cambridge advocate that SERC should establish a centre for astronomy in Cambridge to which a substantial part of the staff from Greenwich would be transferred. One of the other contenders is the University of Manchester, whose case is based on the commonality of interest between the radioastronomers of Jodrell Bank and those at Edinburgh concerned with the millimetre telescope on Hawaii.

These arguments will presumably be adjudicated by the committee under Sir John Kingman, chairman of SERC until last October, which is looking into the future of the two observatories.

The emergence of these proposals is an illustration of the difficulties faced by SERC in trying to match its future organization to its expected budget. Its plan is more a restatement of familiar problems than a resolution of them.

The starting point is the council's statement that its work is at present limited by the shortage of funds, with the result that many good projects cannot be supported. SERC on this occasion points to increasing competition in basic research not only "by the USA and Japan, but by France and Germany". The plan argues that science has "an exponential dynamic", implying that discovery creates new needs, and says that external constraints on its operations at present are "too severe".

For the immediate future, SERC says

that it hopes by 1989 to release an extra £18 million a year from its own resources. The most urgent use of the funds, the plan says, is for the direct support of universities. As well as seeking a reduced subscription to the CERN laboratory at Geneva, SERC intends to reduce spending on support for high-energy physics by a fifth. On space research, the council says that it will concentrate its support for the European Space Agency on projects in basic research, and that its intended participation in the British National Space Centre will not persuade it, "at least for

the time being", to increase spending.

Among the other casualties of planning within restraint are the proposed upgrading of the tandem accelerator at Oxford. Ultimately, SERC says, it will also want to reduce its involvement in the Institute Laue - Langevin at Grenoble, although it hopes before then to have reached agreement with other European research councils for international participation in the Spallation Neutron Source at the Rutherford Appleton Laboratory in exchange for British participation in the European Synchrotron Radiation Facility. □

British research

A little more all round

SIR Keith Joseph, British Secretary of State for Education and Science, this week announced the science budget for the next financial year (see table). Sir Keith has accepted the advice of the Advisory Board for the Research Councils (ABRC), which each year tells the government how it thinks the money

Government expenditure on British research (£ million)

	1985-86	1986-87	Increase (per cent)
AFRC	50.3	52.7	4.7
ESRC	23.6	23.6	0
MRC	122.3	128.3	4.9
NERC	67.3	70.3	4.5
SERC	298.0	315.5	5.9
British Museum (Natural History)	16.2	17.2	6.2
Royal Society	5.9	6.4	8.5
Others		0.6	
Total for 1986-87		614.6	

should be divided. The only part of ABRC's advice that Sir Keith rejected was that the Department of Health and Social Security should bear the full costs of establishing a centre for coordinating epidemiological research on acquired immune deficiency syndrome (AIDS). Although the health department may pay up to £0.3 million to the centre, Sir Keith has firmly told the Medical Research Council (MRC) to meet the balance of the costs of the centre from its allocation.

The total science budget for 1986-87 includes an extra £15 million that Sir Keith pulled out of his hat last month. ABRC has recommended, and Sir Keith agreed, that of the new money, the Agricultural and Food Research Council (AFRC) should receive £2.5 million for "new initiatives in its institutes", MRC £2.5 million for research grants and programmes, the Natural Environment Research Council

(NERC) £1.9 million, the Science and Engineering Research Council (SERC) £6 million to support "strategic research of industrial relevance" and the rest will enhance ABRC's "flexibility margin".

ABRC intends to produce a strategy document next year to take account of the corporate plans of each research council (see above). It will also change its normal schedule so that it will consider both the overall financial position of the science budget and the relative positions and claims of the individual research bodies in one exercise in March 1986.

The only body not to receive an increase in funds for 1986-87 in Sir Keith's statement is the Economic and Social Research Council (ESRC). The council is unlikely to be too distressed at the news; this is the first time in four years that its budget has not been cut. ESRC has taken seriously its brief from ABRC to toughen up; last month it took the controversial decision to blacklist any institution in which ten per cent of research students failed to submit a thesis within four years, and deny them the right to apply for any more grants for two years. Fourteen such institutions were blacklisted and given until last week to appeal. ESRC has now considered those appeals, and this week announced that five institutions have been reinstated for various reasons, including inaccurate statistics, failure to inform ESRC of withdrawal and two administrative errors.

Kings College London (KQC), Leeds Polytechnic, the University of London Institute of Education, University College of Wales (Swansea) and UMIST have gained reprieves. But Queen's University Belfast, the Universities of East Anglia, Liverpool, Aston and Dundee, Manchester Business School, Sheffield Polytechnic and Paisley College of Technology have failed to persuade ESRC to reverse its decision.

Maxine Clarke

Strategic Defense Initiative

Congress questions UK pact

Washington

DESPITE high hopes in Europe that President Reagan's Strategic Defense Initiative (SDI) might lead to thousands of millions of dollars of SDI contracts for European scientists and engineers, the Europeans are unlikely to see much more than about \$300 million in total, according to congressional testimony last week by John Pike of the Federation of American Scientists, an unofficial pressure group. Pike's analysis, based on an examination of existing SDI contracts, identifies six major barriers to European cooperation and warns that the political repercussions due to failed expectations will reduce the already hesitant and patchy support for SDI in Europe.

SDI is expected to let contracts worth in total about \$30,000 million in the five years to 1990. But almost half of this will be off-limits to European countries because of articles and "agreed statements" in the Anti-Ballistic Missile (ABM) treaty of 1972, which prohibit transfer to other states of not only ABM systems and their components but also technical descriptions and blueprints specific to such systems. The US administration has repeatedly said that it is conducting SDI research in full compliance with the ABM treaty, and British support for SDI is conditional on continuing compliance.

Pike believes the treaty will prevent European participation in major SDI projects such as sensors, rocket interceptors and directed-energy weapons. Instead, participation would be restricted to basic research and development only of very small devices and subsystems, for which the dollar value would be "trivial".

Pike's second major impediment to cooperation is the limited capabilities of European companies, which in his view puts a further one-third of SDI beyond their reach. Pike pointed out that, in contrast to the very limited experience of European companies, the United States has spent \$50,000 million on ABM research over the past 30 years.

A twelve-year-old US defence procurement regulation prohibits contracting with foreign sources if a US source is equally competent and willing to do the work; exceptions appear to be allowed only if the foreign government concerned reimburses the US government for the work. The record so far is not encouraging: none of the 1,000 SDI contracts so far let have been to non-US companies (there is one small subcontract in Britain) and there were similarly no contracts for non-US companies among the \$3,000 million spent by the US Army on ballistic missile defence between 1975 and 1985.

Having disqualified non-US companies from \$24,000 million of SDI work on treaty and technical grounds, Pike went on to tell a subcommittee of the House of Re-

presentatives' Committee on Banking, Finance and Urban affairs that a further \$5,000 million was excluded because its commercial potential would mean keeping it in the United States or because of inherent geographical restrictions. Of the \$1,000 million left over that would be available for foreign competition, Pike estimated that a maximum of 30 per cent is likely actually to go overseas.

The hearing was held shortly after the announcement of the signing of a memorandum of understanding between Britain and the United States on SDI. As the terms have not been made public, the likely effect of the agreement is hard to gauge. But Representative John LaFalce (Democrat, New York) told Dr Gerold Yonas, chief scientist of SDI Organization, that he believed the purpose of the US administration in obtaining the agree-

ment with the British was political in that it was expected to foster European political goodwill. LaFalce warned that the United States might "build itself up for a fall" if the agreement created false hopes.

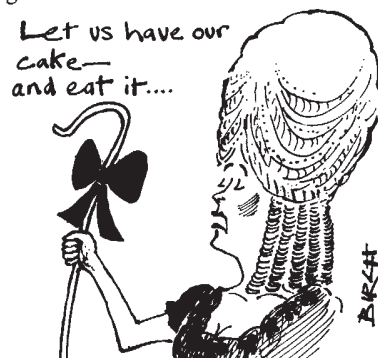
Concern about the effect of the agreement on US employment and the economy was evident in aggressive questioning of Yonas by Representative Bruce Vento (Democrat, Minnesota). Vento repeatedly asked how there could be open competition for research contracts if even a non-specific commitment had been made to support research in Britain. LaFalce questioned whether the United States should be entering agreements that might mean US taxpayers' money generating commercial spin-offs to benefit Europe. Questions were also asked about the confidence the administration had that classified material would be kept secret in Britain. Yonas replied that one of the purposes of the intergovernmental agreement was to control classified information centrally.

Tim Beardsley

European military technology

France veers towards integration

THE US Strategic Defense Initiative (SDI) is an imperfect shield in response to which the Soviet Union need only "sharpen its foil", French scientists claimed this month at the second open "science and defence" meeting organized by the French government.



But the canny French do not ignore the implications of the US programme. Did they not launch the Eureka programme of European collaboration in high-technology products in response to SDI? Last week, M. Roland Dumas, the French foreign minister, said that Europe should now also pay attention to the development of a military counterpart to Eureka. Dumas thus mildly echoed the somewhat stronger proposal a week before of the previous President of France, Valéry Giscard d'Estaing, that Europe needs not only a military Eureka, but its very own "star wars" shield.

Dumas did not go so far. Nor did the other participants in the meeting, held at the Ministry of Defence's own grande école, the École Polytechnique. The technical aspects of SDI were thoroughly rehearsed — a defensive satellite system

would need twenty times as many satellites as could be used to repel an attack, 95 per cent of the system being likely to be out of range, while the Soviet Union could launch missiles in convoys to saturate this usable 5 per cent. The hardening of launchers, a reduction in the boost period, rotation of the vehicles and other measures could increase the power requirements of directed-energy weapons twentyfold, people argued. And cruise missiles deep in the atmosphere could avoid the system altogether.

Dumas said that SDI has nevertheless already achieved one thing — the "scattering" of Europeans, with each nation reacting differently to the challenge both technologically and politically, despite the counterbalancing effects of Eureka. There must in particular be an effort at cooperation in military technology within Europe, Dumas said, overlooking the recent commercial and diplomatic conflict between France and Britain over the supply of advanced battlefield communications technology to the US Army. Dumas went on to say that it is time "to provoke a political effort among governments . . . very soon in the coordination of military and industrial policy". Dumas was arguing, indeed, for a truly European "military/industrial complex" of the kind that has stimulated much basic and applied research in the United States and which, so far, is lacking in Europe. Strange again, perhaps, for the foreign minister of a Socialist government which in rosier days was arguing for the banning of nuclear tests and a curbing of the world arms trade, of which France is now the leader behind only the United States and the Soviet Union.

Robert Walgate

Japanese ethics

Edging towards transplants

Tokyo

HEART transplants are likely to begin in the near future in Japan following the preparation of a new definition of "brain death" by an advisory panel of the Ministry of Health and Welfare. Only one heart transplant has ever been carried out in Japan: that was 17 years ago and caused a major outcry, including demands that the doctor responsible be tried for murder. Similar demands for prosecution were again made this year when surgeons performed a combined liver and pancreas transplant operation at Tsukuba University.

One major factor that facilitates opposition to transplants is the lack of an officially recognized or widely accepted definition of "brain death". A patient who is brain dead has lost all brain activity and has no possibility of recovering. But the use of "artificial lungs" and drugs to maintain blood pressure can keep the patient breathing and his heart beating, often for periods of many weeks. Transplants of "living" hearts and lungs from these patients can save other people's lives.

Without continuous artificial respiration, a brain-dead patient's heart will stop very quickly; a patient whose brain stem still functions and who can breathe without artificial aid is a different matter. Whether or not the life of these latter patients can be legally terminated has recently been the focus of attention in the West. Brain death has, however, already been accepted as a legal criterion of death and has made transplants of heart and liver almost commonplace. Japan on the other hand is still a long way from accepting brain death as death of the person.

Until now, a set of criteria of brain death recommended by the Japanese Society of Electroencephalography has been in general use but each hospital has modified it as it sees it. There has been general reluctance to remove organs from brain-dead patients in order to conduct transplant operations; instead, patients have been maintained for weeks or months until the heart stops beating and the patient is recognized as legally dead. This has both inhibited the performance of transplant operations and tied up expensive medical facilities.

The new definition is considerably more straightforward than the earlier one. It was worked out by a panel of eight neurologists who examined the medical records of more than 700 cases of brain death. Five symptoms are now taken to define brain death: deep coma; loss of spontaneous breathing; enlargement of both pupils to more than 4 mm; the loss of reflexes governed by the brain stem; and flat brain waves, all lasting for at least six hours. Certain cases are excluded — those involving young children whose powers of recovery may be exceptional and those

caused by poisons.

Armed with the new definition, doctors will have a much clearer guide to the propriety of recognizing a patient as dead and the number of transplant operations is likely to increase. But the new definition is only a beginning and by no means clears away all obstacles to transplant operations. The ministry has no intention of taking the next step and preparing a new law that would recognize brain death as a legal definition of death. Doctors responsible for brain-dead patients will thus still be in an ambiguous position and acceptance by family members will be required before brain death can be agreed to constitute the death of an individual. The extent to which brain death is truly recognized and the facility with which transplants will be carried out will thus depend on how quickly brain death is accepted as "death" by ordinary citizens. At present, surveys show that less than a third of the population are ready to take this step.

One obstacle is a suspicion that doctors

are over-eager to perform transplants. Partly to allay such fears, two doctors on the advisory panel involved in transplant research resigned before its conclusions were released. It was thought that those known to favour transplants would not influence the panel's conclusions.

A separate difficulty lies in public attitudes towards organ donation. Despite figures that show that Japan has fewer believers in religion than any other non-communist country (if belief in some form of after-life or reincarnation can be taken as a definition of religion), there remains in Japan a peculiar resistance to donating organs. Even kidneys are in short supply, although transplants may be made from patients whose hearts have stopped beating. Quite why this is so, nobody is sure; commentators point to everything from the influences of Buddhism and Confucianism to a lack of public information campaigns (see *Nature* 313, 338; 1985).

Several competing hospital groups are known to be eager to boost their reputations by performing the nation's "second" heart transplant, and the new report will certainly improve their chances of doing so without censure.

Alun Anderson

Space telescope

Discretion dispute on viewing time

Washington

US ASTRONOMERS are divided over a proposal to give scientists at the Space Telescope Science Institute (STSI) preferential treatment in the competition for viewing time on the Hubble Space Telescope. The proposal would ensure that principal investigators at STSI got at least 5 per cent of viewing time by using some of the "director's discretionary time" set aside for maintenance, calibration and unforeseen "targets of opportunity".

The proposal has been formally presented by STSI staff to several space telescope advisory committees. The preferred option is that up to half of the director's discretionary time (which amounts in all to 10 per cent of observing time) would be used by STSI principal investigators who failed to win time in the open competition for observing time. Only as much discretionary time as necessary to bring STSI principal investigators up to 10 per cent of the total observing time would be allocated in this way.

Concern about the proposal has been expressed in a letter sent to Dr Lyman Spitzer Jr, chairman of the Space Telescope Institute Committee, and signed by Dr Jeffrey Linsky, chairman of the Astronomy and Relativity Management and Operations Working Group. The working group says the STSI proposal is contrary to the spirit of the initial agreement that led to the establishment of STSI. The letter goes on to acknowledge that the proposal would solve a morale problem at STSI but questions whether the suggested

figures adequately reflect the proportions of competent investigators at STSI and elsewhere. At many other national astronomy facilities, in-house scientists compete in the usual way and succeed in getting an adequate proportion of viewing time, from 15 to 40 per cent of what they ask for.

Linsky's committee also disputes that the proposal would not delay observations by other guest observers. It seems likely that some of the special capabilities of the telescope, for example the ability to track moving objects such as a detail on a planetary moon, will not be completely calibrated at launch, and all of the director's discretionary time may be needed for that purpose.

The reason cited by STSI to support preferential access is that it would be in the general interest of the project to ensure that some scientists at STSI are also observers. STSI argues that its scientists are at a disadvantage in the competition for viewing time because they are working flat out to complete STSI's data handling and control systems in time for a scheduled August 1986 launch and so have been unable to devote enough time to preparing observing proposals.

Dr Riccardo Giacconi, director of STSI, said that while STSI scientists nominally spend 50 per cent of their time on research, in practice many spend only 20 per cent. Giacconi said a system similar to that being proposed is used at Kitt Peak National Observatory.

Tim Beardsley

Electron-beam lithography

How to make chips to order

Menlo Park, California

THE Stanford Research Institute (SRI) International, the commercial contract research institute, claims to have carried the technology of electron beam lithography to the point at which the technique can be used for making individual semiconductor microchips, not simply the masks used in semiconductor mass production. Those responsible say that the technique has hitherto been too slow to be used in mass production, where optical lithography remains the chief technique. But SRI will need a commercial partner — and millions of dollars — to commercialize its machine.

According to Dr Ivor Brodie, director of SRI's physical electronics laboratory, the growing demand for bespoke microchips, both in silicon and gallium arsenide, has helped to make electron beam lithography a commercial proposition. SRI is now looking forward to a market that could be worth \$1,000 million a year by 1992. Brodie says that the demand for bespoke chips was not foreseen. "People want a particular integrated circuit for a particular purpose, and do not want others to copy it", he says. Instead of relying on general-purpose devices programmed for particular functions, he says, "people are now doing the programs, as it were, by hard-wiring".

The SRI machine is certainly quick. A semiconductor wafer 200 mm in diameter, and typically carrying 1,200 separate chips, can be "written in about a minute", according to Brodie, who says that with existing electron beam machines, it takes between half an hour and an hour to "write" a mask of the same size.

The electron beams in the SRI design are fired from an electron gun, focused through a screen of lenses at zero volts to the wafer being processed, which is itself maintained at a higher voltage. The multiple beams can be switched off and on by adjusting the voltage. By the use of several beams, it is possible to etch an identical pattern on several different parts of a semiconductor wafer, thus allowing several identical integrated circuits to be created simultaneously.

NASA Lewis Research Center and the Naval Research Laboratories have led SRI to develop custom-made microchips that could miniaturize CRTs. Capp Spindt, senior research engineer leading the project, says that NASA's interest was originally inspired by the need for a source of high current density for its satellite communications. Thermionic emission on cathodes were inefficient, needing heat to excite electrons from an element into the vacuum to create the electron beam. SRI's approach is based on field emission techniques in which electrons are produced by tunnelling to the surface of the metal on which is induced a high

electric field. The process is efficient.

Using this foundation, SRI has developed microchips which produce very-high-current densities — more than 60 A cm⁻² to 100 A cm⁻² or about three times the current densities normally expected of thermionic emission. The design, says Spindt, is based on the well-tried methods adopted for years in the semiconductor industry — a sandwich of metal, silicon oxide and silicon (MOS).

Using electron beam lithography, the researchers were able to etch out the design creating a pattern of holes in the substrate into which they could insert a metal, the source of electron emission. "You have this metal film with a hole in it. You

have an insulator spacing it . . . and the ground plane silicon you can work from. And it's very closely spaced. Then we developed a technique for growing a very sharp point which rests on the silicon."

The distances between these holes are about 2 µm, giving densities of more than 1.2 × 10⁶ tips per cm², and arrays with more than 10,000 tips have been fabricated. Very high current densities result when the device is electrically excited. The next stage in the development is to explore how the technique can be used to enhance the designs of displays. The high current densities are expected to make any CRT picture brighter — and therefore ideal where the environmental light is at a high level, as in a cockpit/flight-deck — and because of the compactness of the silicon, to reduce substantially the width of any display.

Bill Johnstone

Creationism

California sells out on textbooks

Washington

EVOLUTIONISTS in California were bitter last week about what they call a "sham" hearing held by the State Board of Education that approved revised biology textbooks for state schools despite "hundreds" of factual errors and weak coverage of evolution. Revisions made by the publishers to take account of criticisms by the state's curriculum commission introduced more errors than they eliminated, according to William Bennetta, who has been working for better treatment of evolution in textbooks.

The revisions were made after the curriculum commission recommended in September that the books, for twelve- and thirteen-year-olds, should not be accepted because of their avoidance of evolution and inadequate treatment of human reproduction and ethical questions. Publishers are anxious not to offend the strong US creationist lobby, which has threatened to sue in California over the

books now approved. California represents more than 10 per cent of the US textbook market.

A spokesman for the state board said that the books had been greatly improved by the revisions. Publishers had threatened that a failure to adopt the revised books last week would have made it impossible for books to be produced in time for the start of the academic year next September; because textbooks are adopted in California on a six-year cycle, a delay could have held up planned improvements to books in other subjects. Dr Bill Honig, state superintendent for public instruction, said he believed the books now to be the best in the country. A final review by a curriculum commission subcommittee would be able to eliminate any factual errors in the books that were identified at the hearing.

Bennetta and his supporters are not impressed with this promise, pointing out that the subcommittee has no biologist among its members. Most of the books were not altered extensively, as the evolutionists thought necessary, and even those that made significant changes avoided open acknowledgement that evolution is the received wisdom, often using such terms as "many scientists feel" as qualifiers.

The evolutionists complain that the revised books were deliberately made difficult to examine (one copy only was exhibited, for 12 working days, in the state capital) in order to minimize the risk that errors and weaknesses would be exposed. Bennetta's favourite example is in *Health Life Science*, published by Heath and Company, where a statement in the original version that "many scientists believe that dinosaurs were the ancestors of reptiles" has been replaced with the assertion that scientists classify dinosaurs as the ancestors of reptiles.

Tim Beardsley

Aleksandrov's fate?

ALMOST nine months after the disappearance of Soviet computer scientist Vladimir Aleksandrov in Spain, the Soviet Academy of Sciences last week said that he had disappeared without trace in Madrid on 1 April on his way home from an international conference of mayors of "nuclear-free" towns. Diplomatic representations had received "no clear reply". The academy did not explain why it had been officially silent for so long. But Soviet unwillingness to discuss the disappearance has, undoubtedly, fuelled rumours circulating in the West that Aleksandrov, co-author of the Soviet nuclear winter computer model, had been intending to defect, but was prevented by Soviet security men.

Vera Rich

French space

Will US Congress fill vacuum?

THE French space agency, CNES, plans to spend more on basic science, lifting the proportion of its funds from 6–7 per cent now to 10 per cent by 1990. But this is only a target, according to research director Isaac Revah last week. One of the imponderables is whether US participation in the joint Topex/Poseidon oceanographic satellite, France's biggest project so far in basic space science, will survive the automatic budget-cutting mechanisms to which the US government is now committed (see *Nature* 318, 495; 1985).

So CNES is hatching contingency plans for Poseidon, the French half of the project. British or Scandinavian participation is being mentioned as an alternative. But the possibility of collaboration with the Soviet Union, for which there are many

CNES precedents, is "certainly not" on. The Poseidon satellite instruments, consisting of a high-resolution altimeter, a troposphere radiometer and positioning equipment capable of fixing orbital positions to within 10–15 cm, have an obvious military interest.

The US half of the project, Topex, has also thrown up problems of secrecy. The purpose of these instruments is to infer ocean currents from measurements of ocean topography. Nominally, the project is a civil enterprise. But the US Department of Defense has slapped restrictions on the details of the instrumentation. France, through CNES, has retaliated by offering the United States only black-box descriptions of what the Poseidon instruments will do. If the US National Aero-

nautics and Space Administration (NASA) fails to get its money, Poseidon will be flown on a French Earth observation satellite, one of the SPOT series, in 1990–91. "We don't need technology transfer in this field", Revah says.

The French space programme for the next few decades, now becoming clear in its outlines, is diverse and ambitious. In basic science, according to Revah, more attention will be paid to Earth science, but not at the expense of astronomy, supported mainly through agreements with the Soviet Union. On the hardware side, France is committed to the development of the Hermes space shuttle, the cryogenic third stage of Ariane, and participation in the European contribution to the US space station, the separate vehicle called Columbus.

For the rest, the following projects and programmes are on the cards.

- A satellite called Magnolia for precise measurement of the Earth's magnetic field, possibly to be built before 1995. (A similar project for the measurement of gravitational fields, called Gradio, has encountered technical snags and may be put back.) Beyond that, CNES prefers to talk of programmes rather than particular satellites, but there is an inclination to concentrate on measurements that will help to define the hydrological cycle in the troposphere, with obvious applications in climatology. By 1995, the officials say, it may also be possible to make direct measurements of the ground motions expected from plate tectonics and at geological faults, either by the comparison of successive high-resolution photographs or with the help of fixed ground beacons.

- In astronomy, priority will probably be given to Franco-Soviet collaboration on Vesta, a mission to Venus and the asteroids, that will cost France FF600 million. But there is a host of other projects, suggested by the French community, competing for CNES funds. These include a low orbiter for the study of the internal structure of Jupiter, a Mars rover that would look for traces of water, a radio-interferometer called Trio and participation in the proposed European Space Agency (ESA) high-resolution spectrographic X-ray telescope. The only certainty is that CNES will not be able to support all these projects.

- On manned spaceflight, CNES hopes to arrange a long-duration space flight with the Soviet Union so as to engage the French space medicine community; as always, it is looking for industrial interest in the exploitation of microgravity environments.

This year, French spending on these activities will have amounted to FF150 million in bilateral programmes, almost entirely on instrumentation, plus FF200 million on the ESA science budget. This makes a total of FF350 million, compared with the goal for 1990 of FF600 million.

Robert Walgate

Soviet science

Making policy by TV camera

A REMARKABLE innovation in television programming, allowing public questioning of Soviet government officials, seems to have been part of the preparation for the Soviet Union's 27th Party Congress in February. On 29 November, Soviet television stations broadcast a phone-in with viewers' questions about "accelerating scientific and technical progress", a keynote phrase of all Soviet economic documents of the past decade.

The panel included Academician Anatoli P. Aleksandrov, president of the Soviet Academy of Sciences, two vice-presidents of the academy (Evgenii P. Velikhov and Yuri A. Ovchinnikov), two deputy prime ministers (Ivan S. Silaev and Gurii I. Marchuk) and the chairman of the State Committee for Discoveries and Inventions, Ivan S. Nayashkov. The producers seem to have created an atmosphere of spontaneity, but since it had also been possible to telephone questions in advance, and since the questioners' voices were never heard, it is impossible to know how many replies were genuinely unprepared.

Nevertheless, the programme does seem to have reflected genuine public criticism and doubts. Here are summaries of some of the exchanges:

Q: Why are the power sets of the Leningrad, Ignalina and Chornobyl nuclear power stations built in five different — and flawed — designs?

A: We're working on it.

Q: Why does it take more than 12 years for a "practical invention" to be introduced into the economy?

A: It takes only six years, and we are trying to reduce this time.

Q: What are personal computers, and won't they make people "unlearn" how to count? (This question was from a school-

boy, presumably too young to have started the new compulsory computer studies course.)

A: On the contrary, they expand the human capacity for thought.

Q: What is being done to overcome "high-handed administration" and "the cult of the director" in certain scientific establishments?

A: The powers of directors are now being widened, but we have the means to counteract the negative features of high-handed administration.

Some questions, however, seem to have been specially picked to allow the panel to expatiate on new plans and proposals — the concentration in the new Five-Year Plan on machine tools, the establishment of biotechnology centres in rural areas or of a laboratory to monitor how the young generation in schools accept computer technology, and "whether it will do any harm to their development".

A question to Academician Ovchinnikov in his role as chairman of the All-Union Society for the Struggle for Sobriety itself answered one question that has been puzzling many Soviet citizens, not to mention Soviet-watchers abroad — why have scientists in particular been coopted in such force into the anti-alcohol movement?

Ovchinnikov's answer is that the acceleration of scientific and technical progress requires increased labour discipline, that is, the elimination of drunkenness. Ovchinnikov, himself, it appears, is in favour of total abstinence.

Other questions related to recent policy decisions such as the merging of the medical and microbiological industries, and the commitment to thermonuclear power emphasized at the Geneva summit.

Vera Rich

British research

Catalogue of steady decline

ALTHOUGH declining British government support for civil research is offset by an increase in support for military research, this has not prevented a decline both of the proportion of the Gross Domestic Product (GDP) spent on research and of Britain's standing relative to other industrial nations in civil research spending. This is the simplest lesson to be drawn from the *Annual review of government funded R & D 1985*, published earlier this week (HMSO, £9.50).

The survey is the fourth in the series produced since the House of Lords Select Committee on Science and Technology recommended in 1981 that there should be such a document. It is also the last to be produced by Sir Robin Nicholson, the scientist member of the Cabinet Office who will be leaving in the new year to become research director of Pilkingtons, the glass manufacturers.

Nicholson obviously hopes that he established a tradition that will survive not only his office but general elections yet to come, explaining how future editions will improve on the quality of the data (which is at present variable, especially on topics such as manpower). One guarantor of survival is that the survey is entirely free of value judgements, and that government departments' own estimates of their research spending under various categories are normally entered as supplied.

The survey is largely based on figures for spending by government departments covering the last financial year (to last April) together with the three immediately previous years and plans for the present and the two succeeding years (as announced last February). The figures, such as they are, for industrial spending on research and development come from a survey carried out by the Department of Trade and Industry.

In the current year, according to the survey, government spending on research and development is at a shallow peak, amounting in real terms (and 1983-84 prices) to £4,137 million. On present plans, the total is expected to decline from now on, to £3,966 million in 1987-88. Within this total, however, defence spending on research and development, now just over a half at £2,077 million, is expected to increase modestly to £2,181 million in 1987-88 (again at 1983-84 prices). But even these figures make the conventional but increasingly doubtful assumption that about 30 per cent of spending on higher education may be counted as research expenditure.

Nicholson emphasized earlier this week that the international comparisons suggested by the survey are made uncertain by the extent to which the data cannot easily be compared. But according to data collected by the Organisation for Econo-

mic Cooperation and Development (OECD), Britain stands out as the only major member country in which total spending on research and development (by industry as well as government) has actually declined in recent years (by 0.9 per cent in 1983, the latest year for which a comparison is attempted). □

US panel split on guidelines

Washington

AGREEMENT was finally reached on 4 December on policy guidelines for the safe introduction of recombinant DNA technology by a working group of the Organisation for Economic Cooperation and Development (OECD). The guidelines have now only to be formally approved by OECD's Committee on Science and Technology Policy before being published.

The drawing up of the guidelines, which has taken two years, spawned dissent between US agencies which led to the cancellation of a meeting of the working group in August and expressions of concern by US senators. At the meeting earlier this month at OECD's headquarters in Paris, the US delegation offered a radically changed version which omitted a section of a previous draft dealing with large-scale industrial practices. The compromise document now agreed represents "a merger of the best of both versions", according to Dr Frank Young, commissioner of the US Food and Drug Administration (FDA), who secured the eventual agreement between US agencies. Dr Henry Miller of FDA, whose involvement in the project generated controversy, said he was "absolutely delighted" with the results.

The confusion over the US position delayed completion of the guidelines by 4-6 months. The representative of the Environmental Protection Agency who earlier coordinated US work on the project boycotted the latest meeting.

The document now agreed endorses the use of agreed standards of good industrial large-scale practice for genetically engineered organisms deemed to represent a low risk; many OECD countries had been waiting for this recommendation to cite as justification for their domestic policies. Higher-risk organisms can be safely handled with established containment techniques. When considering environmental releases of genetically altered organisms, the document says risks can be evaluated in the same way as for other organisms, and recommends a step-by-step approach of progressively expanding the containment through small-scale and then large-scale field tests before allowing full release.

Tim Beardsley

Deep-sea drilling

Britain rejoins programme

A BLACK sheep became a prodigal son last week, when the British Natural Environment Research Council (NERC) announced that it would be able to find the annual subscription for the new Ocean Drilling Program being organized by the National Science Foundation in the United States through the intermediary of JOIDES, the consortium of US oceanographic institutions responsible for the earlier IPOD (international phase of ocean drilling) programme. Britain participated in the planning of ODP, but did not become a full member because NERC could not pay the \$2.5 million annual subscription.

Signs that the prospects were good were seen last month, when the secretary of the council, Dr John Bowman, said that he hoped to have raised the subscription for 1986 by the end of the year. Now, it seems, the Department of Energy and other government departments have agreed to meet a fifth of the cost, with the oil companies providing 14 per cent. NERC will provide the remainder, equivalent to about £1.2 million at present exchange rates and will also stand the risk of future currency fluctuations.

Everybody seems cheerful about the prospect. At a meeting of the British coordinating committee last Friday, Dr Tim Francis of the Institute of Oceanographic Sciences was appointed as the British representative on the ODP planning committee, next due to meet at the end of January in Hawaii.

Representatives were also nominated for the dozen or so working groups of ODP, now mostly organized on regional lines, but it may be necessary to change these assignments, if, as seems likely, the structure of ODP is recast.

Even so, Britain will participate only in Leg 107 of the new programme, which is planned to sail from Malaga for several drilling programmes in the Mediterranean on 26 December next year. (The first leg of the new programme will put to sea on 31 January 1986.) There is particular British interest in Leg 14, in the Weddell Sea, in the Antarctic near the British station to be drilled in 1987.

Domestically, the British coordinating committee under Professor M.G. Audley-Charles (University College, London) will expect the British representatives on the ODP working panels to act as convenors of parallel groups among British geophysicists. The presence of the oil companies among the sponsors of the British contribution is reckoned to have been politically if not financially crucial, given the British government's belief that industrial sponsorship of research is a public good in itself. □

Japanese energy

Go-go go for nuclear power

Tokyo

JAPAN'S white paper on nuclear power released this week is full of the boundless enthusiasm for nuclear energy that seems to have evaporated in the West. The call is for more nuclear power stations, the rapid development of an independent capacity to process and enrich nuclear fuel and increased effort to develop fast-breeder reactors.

The logic is simple enough. At present, nuclear power stations provide Japan with its cheapest source of electricity. According to the report, this year nuclear-generated electricity cost 13 yen (\$0.6) per kwh, against coal-generated electricity at Y14 per kwh and liquid natural gas generated electricity at Y17 per kwh.

The calculation for nuclear energy does not, however, include the price of decommissioning old nuclear reactors; adding this in gives nuclear power just a very slight edge over coal. Thirty-one nuclear reactors in Japan are between them supplying 23 per cent of the total electric power. That places Japan fourth after the United States, France and the Soviet Union in its total nuclear power generating capacity.

Japan's situation is, however, unique, for the nation has almost no fossil fuel reserves and must import 90 per cent of its energy, much more than any other large industrialized country. In the report's view, the cost of those fossil fuels is bound to rise over the next decade and the advantages of nuclear power become ever more apparent, although oil prices are now falling as the OPEC nations try to boost sales.

The key to Japan's cheap nuclear power is operating efficiencies that are the highest in the world. As nuclear fuel costs are small, the price of nuclear-generated electricity stems from the cost of plant construction and running and thus the percentage of time for which it is in operation. In Japan, power generation is suspended fewer than 0.1 times per year per reactor, just a tenth of the frequency of reactor troubles in the United States and attributed by plant operators to the superior training and conscientiousness of Japanese staff.

Enthusiasm for nuclear power seems to be shared by citizens too, most of whom, according to a government survey, think more electricity should be nuclear generated. Indeed, nuclear power seems to have a rather good image; when those surveyed were asked to list the uses of nuclear power, treatment of cancer was listed second after power generation.

As a government document providing a source of information for decision makers, the white paper might perhaps have painted a slightly less rosy picture. One proud boast of the government is that since commercial reactors began operation in 1966, "there have been no accidents or

failures in which operators or people living nearby were affected by radiation". That, strictly speaking, is true, but it avoids mention of the leaks on board the nuclear ship *Mutsu* and the 1981 leakage of radioactive water from a storage tank into the sea. Nor will its simple description of fast-breeder reactors as the mainstream power source of the future "fundamentally solving the problem of nuclear fuel sources" appeal to many, including those in Japan, who have tried to estimate the likely economics of these reactors.

Alun Anderson

Polish science

Congress delay

THE third congress of Polish science, scheduled for 9–10 December, has been postponed until March 1986, due to the "need for more thorough preparation of the plenary reports and also the papers being completed by the 16 problem teams". The purpose of the congress had been to review Polish science with special reference to the research applications in the economy.

The old hierarchy of graded levels of priority for research problems from "government" and "key" problems down to "departmental" problems, introduced after the second congress in 1973, has proved unworkable; some new research structure is clearly necessary. But it is apparently proving difficult to devise a viable alternative.

The first congress of Polish science (June 1951) served not only to assess the state of science but also to impose a rigidly Marxist philosophy on Polish research and learning. There are widespread fears (particularly after last month's dismissals of university administrators) that the forthcoming third congress might launch a similar purge.

Although the official reports for the congress may not be ready, one unofficial report has already appeared in the underground press. This is a 10,000 word pamphlet from the unofficial "Social Commission for Learning", which attempts to outline the effect of the crisis and martial law on Polish science and learning, including the "reorganization" of the Institute of Nuclear Research, the run-down of investment in science, the cessation of doctoral research in the universities in 1982 (it has only partially been restored), and in particular, the situation in the 200 or so research institutes responsible to some ministry or government department rather than to universities or the academy.

The pamphlet, although written in a detached and academic manner, gives a depressing picture of Polish science.

Vera Rich

AIDS

Pasteur sues over patent

Washington

THE Institut Pasteur in Paris has filed suit in the US claims court in Washington DC, contending that researchers at the US National Cancer Institute (NCI) used Pasteur's specimens and data to develop and patent a commercial test to detect antibodies to the AIDS (acquired immune deficiency syndrome) virus. The suit asks the court to declare that Pasteur researchers Luc Montagnier, Jean-Claude Chermann and François Barre-Sinoussi were the first to isolate the AIDS virus and to recognize its significance in developing blood tests for AIDS antibodies.

Institut Pasteur researchers were the first to publish an initial characterization of the AIDS virus and filed a patent application for a test in December 1983; the application remains pending. The NCI researchers, led by Robert Gallo, filed a similar application in April 1984 and received a patent 13 months later.

Montagnier has claimed that he sent his viral isolate to Gallo as a scientific courtesy for research purposes in July and September 1983. But Gallo says the rate of production of virus in the cultures he received made them useless practically, and that development of a test and reliable characterization of the virus was impossible until his laboratory found a cell line that could produce the virus in quantity.

Montagnier's original isolate has subsequently been found to be very similar to Gallo's, but Gallo angrily rejects suggestions that his isolate was in any way derived from Montagnier's. Gallo says other isolates have been found that are both more and less similar to Montagnier's than his original, and that the similarity is nothing more than might be expected given that both originated from patients in New York City.

Gallo's patent, which was turned over to the US government, forms the basis of the tests currently in use in the United States for AIDS antibodies. The commercial value of the patent is counted in millions of dollars.

Other tests are expected to enter the market shortly that are derived from the Pasteur isolates, and one manufacturer, Genetic Systems, has said it will not be paying royalties on its test to the US government.

Pasteur had been negotiating unsuccessfully with the US government for the past four months to reach a compromise agreement on royalties from existing tests, which it believes should be shared equally between the US and French institutes (see *Nature* 317, 373; 1985). It appears that the question will now be settled in court: no date for a hearing has yet been fixed.

Tim Beardsley

The cause of university education

SIR—The way in which the political ideology of a democratic government can make or mar the cause of higher education or university autonomy is best illustrated in the action of the governments of France (*Nature* 315, 172–173 & 316, 5; 1985) and Britain (*Nature* 315, 265; 1985). In France, the present government has succeeded in freeing the universities from the overbureaucratization of higher education. The granting of absolute independence to the Comité National d'Évaluation des Universités (CNEU) so that it can make an objective evaluation of a university is a step in the right direction. This may free the university from day to day interference from political or bureaucratic authority. Furthermore, CNEU wants to lay equal emphasis on research and on teaching by individuals and groups within a university. This is most certainly a radical change from current practices in French universities, where too much emphasis has been laid either on research or on teaching in granting promotion to a member of the faculty. Promotion has depended on an individual's local political influence over the central machinery responsible for promotion.

The trend in British higher education has been the opposite. The present British government has inflicted maximum damage on the cause of university autonomy, of which the British have always been proud. Much has been written on the subject in editorials in *Nature* during the past two years. *Nature* has highlighted in a systematic manner the callous disregard of the present government for the sanctity of higher education. In this connection, the address delivered by Lord Attlee on the occasion of conferment of the Honorary Degree of Doctor of Laws by the University of Glasgow in 1951 is worth quoting:

"It has always been one of our proud boasts that our universities are free and are rightly jealous of any attempt by the State to extend its power over them. The administration by the University Grants Committee is a characteristic British device which while it passes muster with the financial critics of the House of Commons, leaves the universities almost complete freedom to run their own affairs. In a democracy it is fundamental that thought should be free and that the inquiring and critical university spirit should be brought to bear on all affairs. The University must ever seek for the truth; it must never be a mere instrument in the hands of a government, a church or any political or economic group."

The respect in which the universities of Great Britain are held is mainly due to the freedom from governmental interference that they enjoy (or used to enjoy before the onslaught initiated by the present government), both constitutionally and actually.

In this crisis, the low-key position adopted by the university community is worrying. British academic institutions have almost surrendered to the political

authority in its attempt to remove the autonomy of the universities. To free us from every kind of domination except that of reason is education in the real sense. This aim can be best achieved in an atmosphere of professional integrity where teachers are as free to speak on controversial issues as any other citizen of a free society. In Cardinal Newman's words, "A university education gives a man a clear conscious view of his own opinions and judgements, a truth in developing them, an eloquence in expressing them and a force in urging them".

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PhD theses

SIR—In regard to Beverly Halstead's cogent remarks (*Nature* 316, 760; 1985) about PhD theses, Linus Pauling must have set the standard when, in 1925, he submitted the reprints of five journal articles as his thesis.

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Greenhouse dilemma

SIR—The background to the "greenhouse effect" is well-known. Carbon dioxide from burning fossil fuels, deforestation and cement manufacture causes global temperature to increase by about 3°C each time the proportion of the gas in the atmosphere doubles. At the current rate of manmade CO₂ production, the proportion will have doubled in about 230 years.

At the end of the last ice age, the global temperature increased naturally by only about 6°C with a corresponding sea-level rise of about 100 metres from melting ice in the following 2,000 years or so. By analogy, this implies a sea-level rise due to the greenhouse effect of at least 12 metres in the next 230 years with a further 38 metres still to come, even if the rate of CO₂ increase remains constant and there is no further rise thereafter. However, there is a close link between atmospheric CO₂ increase and population size.

It seems inevitable, therefore, that the greenhouse effect will induce sea-level rises high enough to drown many if not most major cities of the world and much of their agricultural hinterlands within the lifetime of the sea defences presently designed at massive cost to protect them. Alternatively, the population size might be contained and then reduced to decrease manmade CO₂ production. This would destroy the pensions and insurance structures of the developed world and family provision for the elderly of developing nations.

We appear to face a dilemma. Social structures will not allow us to reduce the population. Current technology and economics will not provide a workable alternative to the consequent continuing generation of CO₂ with its associated sea-level rise and massive incursions.

If we accept the greenhouse calculations, should we not be embracing their conclusions more urgently in the planning of relevant engineering and financial structures? On the other hand, if we choose to disbelieve the warnings, why are we paying dearly for them with public money?

BRUCE DENNESS

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Metric system

SIR—Having grown up in the United States and lived in Britain, as well as visiting and living in several metric lands, I think A.A. Berezin (*Nature* 317, 762; 1985) has missed the point of D.C. Jolly's rather sensible letter on the metric system. A measurement system must first of all be a means by which one can orient oneself to the physical world. Any system satisfying this condition is, in that sense, as adequate as any other. I do not need to know how many square feet there are in an acre; I do need to know that an acre is a generous lot for a house but insufficient land to farm. A mile does not mean 5,280 feet to me. It means a long walk or a short drive. In my laboratory, I use the metric system exclusively, not because I believe it necessarily better but because it is the means by which I was trained to orient myself on scientific matters. When I do a physical examination of a patient, I measure and weigh in metric but report the results to the anxious human in feet and pounds. If Berezin ever told an American mother her baby had a temperature of 37 degrees, he would quickly understand my meaning. For myself as well, centigrade provides too narrow a gauge to describe bodily comfort compared to Fahrenheit, and also a fever of 104°F has a zing to it that 40°C could never match. The comforting security in tradition can sometimes more than compensate for the inconvenience of technical inefficiency.

Being universal, science needs a universal idiom and if that mandates metric, so be it. But how one sees one's personal world and describes it to others is quite another matter. For those purposes, the British-American system of measurements suits me and millions more just fine. What divides nations and peoples is not how they measure things but intolerance for the ways of others.

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Calculating the orbits of comets

The return of Halley's comet may have been a disappointment, but new-found skill at calculating cometary orbits may help towards an understanding of these objects.

THE COVERT return of Halley's comet has been a great public frustration that may yet send hordes of people scurrying off to the South Pacific in March and April, after the perihelion passage predicted for 9 February 1986, just six weeks from now. In reality, the likelihood that very few people would ever see the comet was well signalled in advance; the Earth is on the wrong side of its orbit to make Halley the spectacular object it seems to have been in 1066 and 1682. At a perihelion distance of 0.59 astronomical units, there can be a tenfold variation in the apparent brightness of the comet with the position of the Earth. At this unfavourable passage, many people will have to be content with reading about the event, in which case they might do worse than browse through the proceedings of last year's International Astronomical Union symposium on the dynamics of comets, which have now been published*.

Comets have an obviously special place in planetary dynamics as naturally occurring test particles in attempts at the solution of the many-body problem in celestial mechanics. Unlike the other objects in the Solar System whose mass is negligible compared with that of planets, they have the particular advantage of moving in orbits that are often far from being circular. Given the interest in spacecraft orbits and the availability of computer power, not to mention the inherent interest of the subject (and even of Halley's comet), it is inevitable that people should have been so busily calculating cometary orbits.

But is that not simply a matter of solving a few differential equations? There are two practical snags. Comets are not ideal test particles, but self-accelerating objects as a consequence of the way in which they lose mass asymmetrically near perihelion. And even the best-known, among which Halley and Encke (found at the end of the eighteenth century) stand out, travel in orbits whose elements are poorly known, at least in comparison with the orbits of the planets.

Running through last year's symposium seems to have been a hymn of gratitude to B.G. Marsden from the Harvard-Smithsonian Institute of Astrophysics for his compilation over many years of an authoritative list of the orbital elements of more than a hundred periodic comets and for having, during the past two decades, founded the small but flourishing school of those who would put the treatment of

the "non-gravitational" influences on cometary orbits on a rational basis.

What began as an empirical attempt to calculate the acceleration (sometimes the deceleration) of the comet from the changes in its orbit, so as to learn something of the physics of the processes responsible, has been rationalized by supposing extra forces which are functions of the heliocentric distance. On this view, comets accelerate by the transfer of momentum from the escaping gases; for the case of Encke, Y.V. Batrakov and Y.A. Chernetenko from the Institute of Theoretical Astronomy at Leningrad now think that the non-gravitational forces can be related to the magnetic index of solar activity, which makes sense.

In practice, the outcome of these calculations seems as satisfactory as could be expected. The assumption that the non-gravitational force on a comet near perihelion is simply a function of the heliocentric distance and, specifically, that it is a power law works well enough. But the idea that the force could be in any direction than that towards the Sun requires that the nucleus of the comet should be spinning, whence the hope that the Giotto encounter next March may directly throw light on the revolution of the Halley nucleus.

Even without these qualifications, the solution of the differential equations of the motion of a comet is far from being simple. One obvious difficulty is that even the comets with very short periods are known accurately only from their brief passage through perihelion, so that integration entails projecting a set of non-linear equations forward for the equivalent of many years from a poorly known starting point. So far as can be told from last year's meeting, computers have made possible several new ways of tackling the problem, which is why it is (or should be) all the more humbling that, at the very beginning of the eighteenth century, Halley had applied methods to the calculation of twenty cometary orbits and that, by the middle of the century, A.C. Clairaut had been able to allow for the effects of Saturn and Jupiter on the orbit of Halley's comet so as to predict its passage in 1757 to within a month.

The neatest, or at least the most ambitious of all the programmes of calculation described last year, is that which yields comet orbits as by-products of a continuing international project to calculate

the evolution of the Solar System as a whole, known as the Long-Term Evolution Project. The project has the virtue of involving not merely people from Western Europe (among whom Italians are prominent), but also at least one group from Czechoslovakia. The objective is that the motion of the principal objects of the Solar System should be calculated almost from first principles. As with other numerical schemes of this kind, among which that of Edgar Everhart from the University of Denver is the best known, the proof of the pudding consists not of the agreement between predictions and future observations, but of agreement between the calculations and past records of the passage of known comets — "postdiction" as it is anachronistically called.

Many of the results really are quite startling, familiar though they may be to those working in the field. Even Halley is not as well-behaved as might be thought. D.K. Yeomans of the Jet Propulsion Laboratory describes the attempts made since Halley's own work in 1705, including that of Bessel in the early nineteenth century and of those this century who have had the benefit of the ancient Chinese records, only to conclude that this long series of perihelia, if used as the basis of strictly dynamical interpolation, foreshortens each cycle of roughly 76 years by about four days. With the discrepancy explained by the transfer of momentum at evaporation of the surface material, Yeomans concludes that the orbit of Halley's comet can have changed only slowly during the past 18,000 years or so, but that there may have been some more profound interaction with Jupiter at some point.

This fits in well with the calculations made of the behaviour of other comets in the past. There is for example, the case of the comet Oterma, whose orbit lies between those of Jupiter and Saturn, which encountered Saturn in the late eighteenth century, which has just spent two decades as a distant satellite of Jupiter, but which is now likely to return to much what its former orbit was. This is one of many examples quoted at the symposium to illustrate how comets filter their way from the supposed Oort cloud of comets-in-waiting, at some distance beyond Neptune, by successive encounters with planets into the inner Solar System. The comets we see are the exceptions.

John Maddox

* *Dynamics of Comets: Their Origin and Evolution* (eds Carusi, A. & Valsecchi, G.B.) (Reidel, Dordrecht, 1985).

Membrane proteins

Structure of a bacterial photosynthetic reaction centre

from Richard Henderson

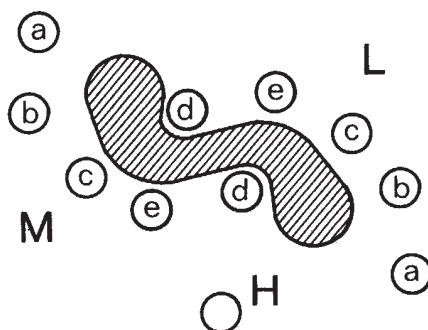
ON PAGE 618 of this issue J. Deisenhofer *et al.* report the first structure at atomic resolution of a protein — or, more accurately, a complex of several proteins — from a biological membrane. Not only is it a superb structure but since it is of a photosynthetic reaction centre complex (from the purple bacterium, *Rhodospseudomonas viridis*), the result provides a structural basis for understanding photosynthesis. In addition, the work bears witness to the tremendous and continuously growing power of protein crystallography.

A great handicap in understanding any of the vital processes carried out by membrane proteins has been the lack of atomic structures for such proteins. The major problem is to release the proteins in undamaged and stable form from the lipid bilayers that constitute the membranes. There is no such problem with the water-soluble cytoplasmic proteins, 200 or so of which have had their structures solved. Ironically, since water-soluble proteins need a non-polar interior and a polar surface whereas lipid-soluble proteins could be largely hydrophobic and their internal hydrogen bonds would not have to compete with the hydrogen-bonding potential of the surrounding water, the architecture of membrane proteins might actually be simpler than that of cytoplasmic proteins.

It was over three years ago that Hartmut Michel produced large well-ordered crystals on the *Rps. viridis* reaction-centre complex using a method somewhat similar to those used to crystallize many water-soluble proteins, but based on a thorough understanding of the behaviour of the protein–detergent complex in the presence of small amphiphiles and of the precipitant, 2.5 M ammonium sulphate (*J. molec. Biol.* **158**, 567; 1982). There then began a fruitful collaboration between Michel and one of the most productive protein-structure groups in the world, headed by R. Huber. The crystals turned out to contain only one molecule of the complex per asymmetric unit, surrounded by solvent that fills more than 70 per cent of the crystal volume. After collection in a darkened cold room of X-ray diffraction data from the native protein and several carefully selected heavy-atom derivatives, an initial map of the structure at 3 Å resolution was obtained in which the chromophores were immediately identifiable (Deisenhofer, J. *et al.* *J. molec. Biol.* **180**, 385; 1984).

Further interpretation of the electron-density map has now allowed all the components of the photochemical reaction centre to be precisely located. The centre

contains four molecules of bacteriochlorophyll-b (BChl), two of bacteriopheophytin-b (BPh), two different quinones, a non-haem iron and four haems, all embedded in a protein that is made up of four polypeptide subunits containing a total of 1,187 amino-acid residues. The crystallographic unit cell of the reaction centre is smaller than that of the icosahedral viruses whose structures have re-



Schematic diagram illustrating a cross-section through the photosynthetic reaction centre of *Rps. viridis* showing the relationship between the 11 trans-membrane helices (5 of subunit M, 5 of L and 1 of H) and the region occupied by the chromophores, which is shown shaded. The helices are all tilted by angles of up to 38° to the direction of view, which is from the periplasmic side. They are therefore much closer together at the cytoplasmic side of the membrane and much further apart at the periplasmic surface than shown here, which is at a level close to the centre of the membrane.

cently been solved (see Harrison, S.C. *Nature News and Views* **317**, 382; 1985), but unlike them it is devoid of symmetry. It is the largest and most complex atomic structure yet determined. It is even more remarkable that the heart of bacterial photosynthesis, which is similar to photosystem II of green plants, should be the first membrane structure to be solved in detail.

Chromophores

All the chromophores in the complex have been precisely located. The quinone sites include both the strongly bound menaquinone (Q_A) which is found in the native structure, and the more weakly bound ubiquinone (Q_B), which was initially absent in the crystals and had to be located by the difference Fourier method after soaking the crystals in solutions of quinones and quinone analogues. The structure and function of the chromophores in the complex have been reviewed by J. Barber (*Nature News and Views* **315**, 278; 1985). All the chromophores except the haem groups of the

cytochrome are related in pairs by a local 2-fold axis which is perpendicular to the plane of the membrane. Thus on the periplasmic side of the membrane, a pair of BChl molecules are found in close contact with one another. They are called the 'special pair' ($BChl_a$), because spectroscopy has shown that they are in contact and that their oxidation is the primary event in photosynthesis.

Two alternative paths of electronically conducting chromophores extend from this special pair to the single non-haem iron atom which is positioned on the opposite (cytoplasmic) side of the membrane: each path consists of the sequence $BChl_a$ – $BChl$ – BPh – Q_A or Q_B –Fe. These chromophores accept excitation energy from light-collecting complexes of protein and chlorophyll and convert it rapidly into a charge separation across the full width of the membrane — approximately 40 Å. An electron is transferred from the (oxidized) $BChl_a$ to Q_B within 230 picoseconds, apparently always via Q_A which, after reduction, transfers an electron to Q_B , possibly via the non-haem iron. (It is not clear why electron transfer does not also proceed along the other pathway, directly to Q_B .) Following charge separation in the membrane, the BChl is re-reduced by the cytochrome at the periplasmic surface, while Q_B , when fully reduced, is protonated at the cytoplasmic surface and released into the pool of free quinone in the bilayer.

Protein

The four polypeptides — L, M, H and a c-type cytochrome — envelop the chromophores. The L and M subunits provide the body of the structure within the membrane. Each forms an open crescent consisting of a curved wall of five transmembrane helices (a–e in the figure). The chromophores are not totally surrounded, so the L–M protein dimer cannot be described as globular. Indeed, since the chromophores are themselves hydrophobic they need not be separated from a lipid environment. Furthermore, BChl must receive energy from BChl in the light-collecting complexes which sit nearby, within the lipid bilayer, and Q_B must exchange with the pool of free quinone in the bilayer. Thus the function may require direct access to the chromophores from the bilayer. For these reasons, the arrangement of hydrophobic protein helices, hydrophobic chromophores and hydrophobic lipid may be rather open.

The transmembrane helices themselves may have three functions. They may surround the chromophores with non-polar side chains designed to hold them in precise relative orientations. In addition, they contribute the single histidines linking the Mg^{2+} of each half of the $BChl_a$ to the protein and the four histidines coordinated to the non-haem iron; two other histidine ligands to BChl and a glutamic acid ligand to the iron are in non-helical

segments of subunits L and M. And they provide an energetically stable conformation for the polypeptide and a smooth exterior facing the bilayer.

Outside the membrane on the periplasmic surface lies the *c*-type cytochrome with its four haem groups arranged in tandem, allowing electrons to jump from one to the other, ready to re-reduce the special pair of BChl. On the cytoplasmic side sits subunit H, of unknown function, but perhaps involved in some way with the function of quinone Q_B and the determination of the mode of interaction with the light-collecting complexes. Both the cytochrome and the H subunit are globular proteins which, although unique in their fold, have structures typical of many water-soluble globular proteins. The H subunit has an amino-terminal hydrophobic tail which forms a single transmembrane helix, much as is presumed to occur in proteins such as glycophorin.

The structure thus provides a detailed and precise three-dimensional model for the events of photochemical energy conversion. It confirms many previous hypotheses based on other methods and allows new propositions to be formulated. For example, since the weak sequence homology between subunits L and M and the proteins D1 and D2 from photosystem II of higher plants is much more convincing when critical residues in the *Rps. viridis* structure are examined, it is now postulated that D1 is homologous to L, and D2 to M, and that D1 and D2 surround the equivalent of BChl in photosystem II of higher plants.

Construction of the X-ray model depended on sequences of the *Rps. viridis* proteins determined by Michel and his colleagues. But the distribution of hydrophobicity in the amino-acid sequences of the same polypeptides in related bacteria had already led to the prediction of a structure containing five transmembrane helices in each of the L and M subunits and one in the H subunit of *Rps. capsulata* and *spheroides*. The qualitative success of these predictions makes one confident that the many other current predictions of the position of helices in membrane-crossing proteins will turn out to be correct, even if they can be made only in broad one-dimensional terms. It now seems likely that α -helices spanning the membrane will form the hydrophobic domains of all membrane proteins and membrane-protein complexes, with the possible exception of those that form very large aqueous channels, where other secondary structures might be preferred.

The open arrangement of the membrane-spanning α -helices in the reaction centre is different from the more tightly packed helices in bacteriorhodopsin (Henderson, R. & Unwin, N. *Nature* 257, 28; 1975), where they have to line a rather more hydrophilic proton channel, and very different from the arrangement in the porins of the outer membrane of *Escher-*

ichia coli, where β -sheets surround an even larger hydrophilic pore (Kleffel, B. *et al. EMBO J.* 4, 1589; 1985).

When more details of the structure become known, it will be especially interesting to see how the redox potentials of the different chromophores and the transfer of electrons between them are regulated by the stereochemistry of their interac-

tions with the surrounding protein. For now, there is much pleasure to be gained simply from contemplating the superb design of a protein that has its body immersed in a lipid bilayer and its head and legs in water. □

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Planetary science

Hidden carbon dioxide on Mars

from Robert M. Haberle

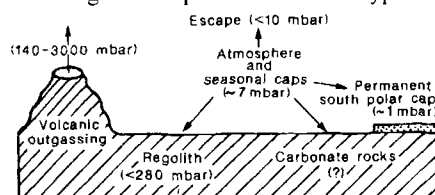
THE atmospheres of Venus and Earth are the primary reservoirs for their major constituents: virtually all of Venus's outgassed carbon dioxide is in its atmosphere, and the same is true for nitrogen on Earth. Mars, however, is different. The amount of carbon dioxide in the martian atmosphere, about 7 mbar, is much less than the planet was originally endowed with, and researchers attempting to sort out the climatic history of Mars are keenly interested to know the fate of the rest. In a recent paper, R. Kahn¹ suggests that much of the outgassed carbon dioxide on Mars is tied up in its crust, in the form of carbonate minerals.

It has long been recognized that the carbon dioxide released into the martian atmosphere may have been incorporated into carbonates². If the total amount of carbon dioxide vented into the martian atmosphere were at the high end of the range of estimates (140–3,000 mbar¹), the idea of a carbonate reservoir becomes particularly attractive for several reasons. First, the polar caps and the regolith, the loosely consolidated rocky surface, which are potentially the largest reservoirs for carbon dioxide other than carbonates, do not seem to have the capacity to hold more than the equivalent of about 300 mbar, so that other sinks are required (see the figure). Second, if Mars degassed large amounts of carbon dioxide early in its history, which is consistent with models of the observed nitrogen isotope ratio³, a significant greenhouse warming would have resulted in surface temperatures that could have been high enough to permit liquid water. This is a leading explanation of the 'runoff' channels found throughout the old cratered uplands in the southern hemisphere⁴. In the presence of liquid water, 1 bar of carbon dioxide could be converted into carbonate rocks in the order of 10^7 years⁵.

Kahn's new suggestion¹ is that carbonate formation on Mars continued after open bodies of liquid water became unstable. With an adequate heat source and sufficient quantities of ice, Kahn argues that transitory pockets of liquid water in disequilibrium with their environment could form; in their presence, carbon dioxide would continue to be converted

into carbonates.

A key quantity in Kahn's hypothesis is the minimum atmospheric overburden pressure, P^* , required for liquid water to form. Below that minimum value, ice would sublime before melting. A reasonable lower limit of P^* is 6.1 mbar, the triple point of water, because with atmospheric pressures below the triple point, liquid water would rapidly sublime. An upper limit is much more difficult to determine but may be as high as 30 mbar, though Kahn gives no detailed calculations to support this value. The most interesting consequence of his hypoth-



Possible reservoirs for carbon dioxide on Mars. Of the 140–3000 mbar of carbon dioxide estimated to have been vented into the martian atmosphere, less than 20 mbar can be accounted for in the atmosphere and polar caps, and lost into space. The two most likely reservoirs for the remainder are the regolith, a near-surface layer of pulverized soil created by meteoritic bombardment, and carbonate rocks, a reservoir created by weathering processes.

esis is that, in the absence of a recycling mechanism for carbon dioxide, the surface pressure of Mars will monotonically decline until it reaches P^* , whatever its value. Kahn believes that the surface pressure observed is very close to P^* .

Before the Viking mission, the surface pressure on Mars was believed to be controlled by the temperature of the permanent polar caps⁶. As the caps represent the solid phase of the atmosphere, the annual mean surface pressure must be equal to the saturation vapour pressure corresponding to their average temperature. But Viking found no evidence for substantial permanent polar caps: the carbon-dioxide frost deposits at the north pole completely disappear during northern summer and the amount that remains at the south pole during southern summer is too small to buffer global pressures, and may even disappear completely in some summers⁷. Thus, it seems unlikely that the

polar caps buffer atmospheric pressure.

Kahn's theory has another implication, apart from the explanation of the low surface pressure. If he is correct, the climate of Mars has evolved linearly over geological time, rather than cyclically. This represents a major challenge to current theories of martian climatic history, which generally invoke a periodic exchange of carbon dioxide between reservoirs in the regolith-atmosphere-cap system to explain the polar layered terrains^{2,5}. The exchange, driven by variations in the orbital parameters of Mars, can result in surface pressures that vary from 0.1 mbar at low obliquity (11.7°), to about 15 mbar at high obliquity (37.1°). These variations are thought to modulate the transport and deposition of dust and water into polar regions, so giving rise to the layered terrains. Over many obliquity cycles, the mechanism would substantially deplete the regolith reservoir and eliminate cyclical climatic changes, because conversion of carbon dioxide into carbonates on Mars is irreversible.

Not surprisingly, there are many uncertainties over the plausibility of Kahn's mechanism. Among them are availability of cations, such as Mg²⁺ and Ca²⁺, for carbonate chemistry; the dependence of P^* on heating and soil properties; and whether or not the favoured regions for carbonate formation could be resupplied with water. It would also be worth knowing just how much liquid water is required. Furthermore, no signature of the vast amount of carbonate that must have been produced has been unequivocally identified. Kahn's discussion of these points is mostly qualitative; the basic physics of the process, therefore, remains to be demonstrated. Nevertheless, it is an intriguing idea that merits serious attention. □

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Geophysics

Transient mantle rheology

from J. Weertman

CONSIDERATION of the ways in which solids can deform leads to the conclusion that the viscosity of the Earth's mantle should increase with depth. This agrees with our current ideas about the thermal structure of the interior of the Earth. Hence it came as a surprise to find that analyses¹⁻³ of glacial rebound and complementary Earth satellite data indicate that the mantle viscosity does not increase with depth but is almost a constant, with a value of about 10^{21} Pa s. Until now, this has presented a dilemma to those studying the physical structure of the Earth and its consequences for plate tectonics but, in this issue of *Nature*, W.R. Peltier⁴ shows that the problem can be resolved if transient creep is taken into account.

The modern study of the creep deformation of the Earth's mantle started with the suggestion⁵ that the mantle might deform plastically through the diffusional transport of atoms between grain boundaries, the mechanism now known as Nabarro-Herring creep^{6,7}. Using the Nabarro-Herring creep theory, R.B. Gordon⁷ found that the mantle viscosity increases with depth. His result is readily understood. The greater the difference between the actual temperature at a given depth and that at which mantle rock first starts to melt, the larger the expected value of the viscosity. The further rock is from its melting temperature the 'colder' it is (even if its actual temperature is quite high) and the more difficult it is to make the rock creep. As the mantle convects, its thermal profile should be approximately

adiabatic. The adiabatic temperature presumably increases much less rapidly with depth than does the temperature at which melting commences and hence it would not be surprising to find that viscosity increases with depth in the mantle.

This conclusion is not dependent on the choice of the creep mechanism which is used to obtain an estimate of the mantle viscosity. Any such mechanism gives faster creep the closer the temperature is to the melting temperature. It also is not dependent on whether the creep mechanism gives rise to a linear, newtonian creep equation ($\dot{\epsilon} = \sigma/\nu$) or to a non-newtonian creep equation such as a power-law creep equation ($\dot{\epsilon} = c\sigma^n$). Here ϵ is the creep rate, σ is the stress, ν is the viscosity and c and n are constants. (For non-newtonian creep an effective viscosity is defined to be equal to $\nu = \sigma/\dot{\epsilon}$. The effective viscosity for non-linear creep requires that a standard stress level or strain rate be specified.)

That the mantle viscosity is almost constant could be explained, of course, if the ratio of the actual temperature over the melting temperature were constant throughout the mantle. It is more likely, however, that this ratio decreases with depth. It could also be explained if mantle creep obeys a power law⁸ and the creep stress increases with depth at a suitable rate. But the glacial rebound stresses decrease with depth. One possible way out of the dilemma is to bring transient creep phenomena into the analysis. In analyses carried out up to now it has been assumed implicitly that only steady-state creep

equations need be considered.

Analyses of isostatic geoid anomalies^{9,10} indicate that the lower mantle has viscosity an order of magnitude greater than that of the upper mantle and hence than the lower mantle viscosity as determined from glacial rebound. But geoid isostasy operates on a timescale of the order of 10^6 years rather than the 10^4 – 10^5 years for glacial rebound. Peltier⁴ investigates whether the order of magnitude difference of viscosity of the lower mantle as deduced from the two phenomena could arise if the glacial rebound method measures a predominantly transient creep strain rate and if the isostatic geoid anomaly method measures a more steady-state creep strain rate.

Peltier makes an explicit calculation by use of a linear creep equation that reduces to a steady-state equation at long times but is a transient equation at shorter times. This equation is for a Burgers's solid¹¹ which, when springs and dashpots model the solid, is simply a Zener standard linear solid in series with a dashpot. For uniaxial stress-strain the equation he used can be written as $\dot{\sigma} + (\sigma/\tau) + (\mu^*\sigma/\nu\tau) = \mu\dot{\epsilon} + \mu^*(\dot{\epsilon}/\tau)$ where the dot stands for time differential, τ is a decay time, μ and μ^* are elastic moduli and ν is the long-term viscosity. Peltier uses this equation only for the lower mantle. For the upper mantle he uses a Maxwell solid (spring and dashpot in series) with the equation $\dot{\epsilon} = (\dot{\sigma}/\mu) + (\sigma/\nu)$. He is able to show that it is possible to reconcile the two values of the viscosity of the lower mantle. He points out that further constraints on the transient creep contribution to lower mantle deformation may require reexamination of the analysis of Richards and Hager¹⁰.

Since Peltier's paper was submitted for publication, a paper by Sabadini, Yeun and Gasperini¹² has appeared in which a transient creep analysis also based on a Burger's solid has been carried out. The results and conclusions are very similar to those of Peltier. Clearly, the study of the rheology of the mantle is entering a new period in which many possible creep mechanisms are being considered as rate controlling, and in which the theorists have become capable of carrying out quite sophisticated studies involving transient creep of mantle rocks. □

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Plant tumours

Wounds activate attackers

from John E. Beringer and Colin M. Lazarus

THE ability of strains of *Agrobacterium* to induce tumours on dicotyledonous plants has excited the interest of most biologists, particularly since it was discovered that tumour formation results from the inheritance of DNA (T-DNA) transferred from the bacterium to the plant, a process that depends on the function of virulence (*vir*) genes. Tumours are induced only where there is a wound on the plant and an intriguing question has been how *vir* genes are regulated so that *Agrobacterium* can recognize wounds and initiate T-DNA transfer. The answer, provided by S.E. Stachel *et al.* on page 624 of this issue, is surprisingly simple: the *vir* genes are induced by specific signal molecules, acetosyringone and α -hydroxy-acetosyringone, that are produced by wounded, but not by intact, plant cells.

This finding helps us to understand why wounds are required and demonstrates yet again how much tumour formation depends on interactions between the two partners. A series of interactions can now be recognized that are logical and all, apparently, directed towards optimizing the efficiency of the interaction from the point of view of the bacterium.

T-DNA is present as part of a large extrachromosomal molecule (plasmid) that also carries a number of *vir* genes. Like other DNA elements capable of transposition, T-DNA is bounded by identical repeats of DNA. Independent loops of T-DNA are found when *Agrobacterium* is cultured in the presence of plant cells and it is assumed that these are T-DNA intermediates in the transfer to the plant cells. Seven genes are required for T-DNA transfer. Two of them, *virA* and *G*, are expressed in vegetative cells but the other five are induced only in the presence of acetosyringone or α -hydroxy-acetosyringone.

Once it is transferred to the plant, T-DNA integrates into host DNA and the genes it carries are expressed. T-DNA encodes genes involved in the synthesis of plant hormones and for the synthesis of novel compounds (opines) that are produced only by plant cells containing T-DNA from *Agrobacterium*. Tumour formation appears to be a method evolved by *Agrobacterium* for inducing the plant to produce a large number of cells that synthesize opines, which are metabolized by *Agrobacterium* but by few other organisms.

Opines have also been shown to act as specific inducers of the transfer (*tra*) genes carried on *Agrobacterium* tumour-inducing (Ti) plasmids. It is now clear that plant metabolites induce the functioning of two distinct sets of *Agrobacterium*

genes: those for virulence and those for transfer of Ti plasmids. In both cases, DNA replication and mobilization leading to gene transfer are induced only when they are likely to be of direct value to *Agrobacterium*.

As well as answering the immediate questions of why only wounded tissue is infected by T-DNA, Stachel *et al.* may also have resolved the enigma of why strains of *Agrobacterium* carrying *Rhizobium* nodulation genes form nodules on the roots of appropriate legumes, whereas they induce tumours when inoculated on wounded tissue; presumably the close association of the bacteria and plant cells during nodule development does not lead to the induction of *vir* genes because the plant is not wounded.

This report is another example of how the study of plant-microbe interactions can yield important information on genetic regulation. Building on evidence that plant defences against pathogenic fungal attack are triggered by fragments of the host plant cell wall, K. Tran Thanh Van *et al.* (*Nature* 314, 615; 1985) have recently

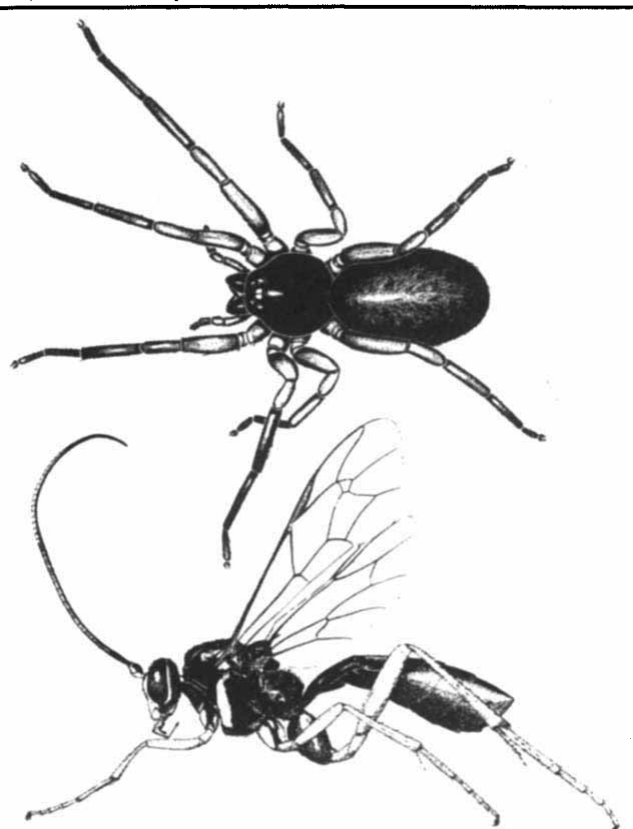
shown that specific oligosaccharides can affect the developmental fate of plant cells in culture. This indicates a role for such 'oligosaccharins' in normal organogenesis. Recently J.T. Mulligan and S.R. Long (*Proc. natn. Acad. Sci. U.S.A.* 82, 6609; 1985) and L. Rossen, C.A. Schearman, A.W.B. Johnston, and J.A. Downie (*EMBO J.*, in the press) have reported that a *Rhizobium* nodulation gene is induced by root exudates in an analogous manner to *Agrobacterium vir* genes. These findings identify primary signalling molecules and raise questions concerning the transduction of signals from the cell surface to the genetic material.

What about interactions such as those of mycorrhizas and between host plants and other pathogens? Progress

in the search for similar inducers may be slow. As Stachel *et al.* have shown, finding an effect of root exudates is only the beginning of a complex isolation and characterization process. To purify the molecules responsible it is necessary to have a good assay, which in general is not an assay of a plant response. Both Mulligan and Long, and Stachel *et al.* took advantage of the extensive genetic understanding of *nod* and *vir* genes, respectively, to produce fusions of these genes with the *Escherichia coli lacZ* gene and thus could determine induction by measuring β -galactosidase activity using a simple and sensitive assay procedure.

We look forward to seeing a surge of activity in the identification of plant molecules that act as specific inducers of genes in symbiotic and pathogenic microorganisms. Perhaps, as with other aspects of plant-microbe interactions, work will be driven by studies of the genetically well-characterized bacteria *Agrobacterium* and *Rhizobium*. It is good to see that studying the biology of tumour formation can yield results that are at least as satisfying as those obtained through molecular studies of DNA. □

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The spider, *Subantarcia* sp., forms the frontispiece to the first formal presentation of the *Orsolobus* taxon of spiders, members of which are found in South America, Australia and New Zealand (Forster, R.R. & Platnick, N.I. *Bull. Am. Mus. nat. Hist.* 181, 1; 1985). The fly is the frontispiece to a taxonomic study of the ichneumon-fly genus *Banchus* in the Old World (Fitton, M.G. *Bull. Br. Mus. nat. Hist.* 51, 1; 1985).

Biophysics

XANES spectroscopy and the crystallographic imperative

from John Galloway

PERHAPS nowhere in the determination of molecular structure is precision more at a premium than in the case of metalloproteins, so that any method that promises to enhance or improve on single crystal protein crystallography is worth investigating. X-ray near-edge structure (XANES) spectroscopy is claimed to represent such promise. The real question, of course, is whether that promise can be realized; in the paper by A. Bianconi *et al.* on page 685 of this issue we might have had the first chance to find out. As it happens, we don't.

What exactly is XANES? Absorption of X-rays takes place chiefly through the photoelectric effect. An electron is ejected from the K shell of an atom (or possibly some other shell). Its chances of escaping are modulated by its being scattered by neighbouring atoms. The resulting fine structure in the absorption cross-section expressed as a function of the energy of the incident X-ray photon contains information about the local atomic geometry. For energies not much in excess of the threshold represented by the absorption edge, say within 50 eV, the photoelectrons are strongly and multiply scattered. These modulations are referred to as XANES.

At higher energies the scattering is weaker and dominated by single scattering events. In this energy regime the fine structure is the rather better known extended X-ray absorption fine structure (EXAFS), which has been widely used in various types of structural study, including some on metalloproteins, since about 1972, the year in which it first became possible to replace the conventional X-ray tube by synchrotron radiation. The domination by single scattering events confers on EXAFS an immediate advantage it shares with a number of other physical probes of structure, namely that it is relatively easily interpreted. But it also confers the considerable disadvantage that it contains information only about two-particle correlation functions, so that only the *radii* of the coordinating shell of atoms and slightly more remote shells in metalloproteins can be extracted.

XANES, by virtue of its strong multiple scattering, contains information about three- and higher-order correlations. Thus, in principle, information about the detailed geometry of arrangements in the shells should be extractable — angles as well as distances. A lot is made of this feature by Bianconi *et al.* The Fe–C–O bond angle is obtained in a single crystal of carboxymyoglobin from the angular de-

pendence of the XANES spectrum, and a difference in this angle between carboxymyoglobin and carboxyhaemoglobin is shown to exist in solution. Unfortunately, the paper gives no indication of the accuracy of these measurements although it is unlikely to be better than $\pm 10^\circ$. On the face of it this seems to compare quite well with the angular accuracy that can be achieved by conventional crystallography but the question is not explicitly addressed by the authors.

That XANES might be interesting at all to structural biologists is a consequence of the two cardinal deficiencies of single crystal methods. First, that they are obviously only suited to crystals, whereas XANES and EXAFS can and do provide structural information about proteins in solution. Second, that in single crystal determinations the extent and quality of the data limit accuracy; limited resolution and phasing errors typically lead to a precision in atomic position of no better than 0.3–0.5 Å, although with great care standard deviations on atomic positions can be brought well below 0.1 Å, which will be needed for understanding function in metalloproteins, where distances between metal ions and their ligands may need to be known to better than 0.1 Å resolution.

Without a good idea of a method's accuracy, realized or potential, no technique will or should be taken seriously. Early proponents of EXAFS rashly made unfounded claims of its superiority over crystallographic analysis of haemoglobin (Eisenberger, J.B. *et al.* *Nature* **274**, 30; 1978) and got a bloody nose for their pains (Perutz, M. *et al.* *Nature* **295**, 535; 1982). But EXAFS has been rehabilitated

through very careful comparisons with crystallographic analysis on 2Zn insulin (Bordas, J. *et al.* *Proc. R. Soc. B* **219**, 21; 1983) and on haemoglobin and insulin (Dodson, G. *Proc. Bioinorganic Discussion Group*, Daresbury, in the press). Dodson concludes that EXAFS has a "clear role in adding precision to the crystallographic picture and hence furthering our insight into the chemical and structural processes that are at the centre of biological phenomena".

Whether the same is true of XANES will be found only in much the same way and will not be easy. It is crucial that the early mistakes made with EXAFS are not repeated with XANES, necessitating rescue operations at a later date. Systematic comparative structural studies of XANES and conventional crystallographic analysis now will pay dividends. The advantage of crystallographic analysis is that production of the electron-density map is not subject to the experimenter's anticipation. XANES is model-dependent — a structure must be imagined and its consequences compared with the experimental data. So the only sound tactic is a heavy investment of effort in alternative structures — a prominent feature of the papers of both Bordas *et al.* and Dodson.

XANES analysis tends to be very heavy on the time of both experimenters and computers. Furthermore, it has inherent limitations, as pointed out by J. B. Pendry (*Comments Solid State Phys.* **10**, 219; 1983). The range of data is small and will not generally be expected to contain many features to be fitted to the model. Indeed, it seems highly optimistic to suppose that the positions of more than three atoms will be found with any accuracy. Therefore, it will not be useful except in harness with other methods and even then its usefulness is likely to lie in choosing between qualitatively different structural alternatives where much is already known. □

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Ecology

Rats as agents of extermination

from Jared Diamond

"TAKE care! Kingdoms are destroyed by bandits, houses by rats, and widows by suitors!" Biologists would emend this insight of the 17th century Japanese poet Ihara Saikaku to note that rats destroy not only houses but also island biotas. The colonization of islands by rats has outweighed all other causes of extinctions of island birds. Rats have also preyed on young Galapagos giant tortoises and exterminated small mammals, large insects, molluscs (Hawaiian achatinellid land snails), and cold-blooded vertebrates (most ground snakes and lizards of Mauri-

tius plus mainland frogs, lizards and tuataras of New Zealand).

Yet it has proved hard to make sense of these gruesome facts. Rats have caused catastrophic extinction waves on some islands, a few extinctions on others, and no visible effect on still others. Somehow, these varying outcomes must depend on the differing susceptibilities of prey species, and on the differing biologies and histories of the three species of rats involved. Ian Atkinson of the New Zealand Department of Scientific and Industrial Research has now made a major contribu-

tion to sorting out this confused situation (in *Conservation of Island Birds* ed. Moors, P.J., 35; International Council for Bird Preservation, Cambridge, 1985). By scrutinizing historical records for the past several thousand years, Atkinson has been able to reconstruct the spread of each rat species. He has thereby established both how the impact of each species differs and the variation in the susceptibility of different faunas exposed to the same rat species. Atkinson's conclusions are not only important to conservation biologists but have also yielded an unexpected insight into the evolution of predator avoidance behaviour.

The three main commensal rat species are the Polynesian rat *Rattus exulans* (here abbreviated *RE*) originally native to tropical southeast Asia, the ship or black rat *R. rattus* (*RR*), from India and southeast Asia, and the Norway or brown rat *R. norvegicus* (*RN*), originally from Siberia and China. These rats reach islands as stowaways on ships. The first expansion (1500 BC–AD 1000) was of *RE*, which reached Pacific islands on Polynesian canoes. From AD 1500–1700 *RR*, by then established in Europe, was spread by ship to islands of the Atlantic and Indian Oceans. By 1716, *RN* had largely displaced *RR* in Europe and was the main rat to ride ships to islands, including some in the Pacific. After 1850, for unknown reasons, *RR* again became the dominant shipboard rat. The spread of rats to islands has proceeded at a fairly steady rate from 1850 to the present, with a peak during and after the Second World War related to establishment of military bases on islands.

With this knowledge of what islands acquired which rats and when, Atkinson then identified the impact of each species. *RE* can climb trees but is the smallest of the three rats and, in terms of documented victims, seems to prey on the fewest species of birds. It may, however, have claimed many undocumented and long extinct species on the Pacific islands during its expansion before AD 1000, and it does still limit the distribution of some invertebrates and cold-blooded vertebrates. (Ironically, the largest bird known to be killed by any rat, the Laysan albatross *Diomedea immutabilis*, is a victim of the smallest rats *RE* — see figure). The largest species, *RN*, climbs trees infrequently and has mainly devastated ground-nesting or burrow-nesting birds, especially seabirds. *RR* is medium-sized but an agile climber and preys on birds at any height from the ground to the canopy. Rats can also have an indirect effect on island biotas by acting as prey for predatory cats, weasels and mongooses, which might otherwise not survive and which also prey on island native species.

In three or four notorious cases, invasions of formerly rat-free islands by rats have rapidly been followed by biological catastrophes. For example, three years after *RR* reached Big South Cape Island (off

Pacific rat approaching (inset) and eating a Laysan albatross. (Photo courtesy Cameron Kepler.)

New Zealand) in 1962, it had reduced or exterminated over 40 per cent of the island's landbird species plus the last surviving population of the southern short-tailed bat. *RR* caused similar catastrophes on Lord Howe Island in 1918, Midway in 1943, and probably Hawaii in the late 1800s. On the other hand, numerous islands with endemic native rats have survived the arrival of *RR* (and sometimes also *RN*) without any subsequent extinctions of birds. This was the case, for example, in the islands of Galapagos, Christmas (Indian Ocean), West Indies, Andaman, Nicobar, New Guinea and the Solomons.

This contrast suggests that birds that have co-evolved with native rats develop predator defence behaviours that allow them to survive invading rats. This, however, is not the sole or even main reason why islands biotas differ in their susceptibility to rats. Many initially rat-free tropical islands (Fiji, Samoa, Tonga, Marquesas, Aldabra and others) have survived colonization by rats without catastrophe. Atkinson notes that land crabs, which are widely distributed on tropical islands, are not only scavengers and grazers, as usually assumed, but also eat birds' eggs and chicks; moreover, some species (especially the giant coconut crab *Birgus latro* and some hermit crabs) climb trees. Thus, they are the invertebrate equivalents of rats, and co-evolution of birds with land crabs may lead to the development of anti-predator behaviour that also protects the birds against invading rats.

Atkinson's analysis has identified new questions. Why did *RR* partly displace the larger *RN* on ships after 1850? Will archaeologists in Polynesia document waves of extinction attributable to the arrival of *RE* with the first Polynesians? What was the original distribution of land crabs? What, specifically, are the predator defense behaviours that are produced by co-evolution with rats, and perhaps land

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

crabs?

But the most important problem is a practical one. Gough, Inaccessible, Nightingale, Clipperton, Snares, Laysan, and some Galapagos islands, to name just a few, remain rat-free. Other islands, including Rennell, Bellona, Rose, Henderson and Little Barrier, have received *RE* but not the deadlier *RN* and *RR*. These islands contain endemic species or relicts that need preserving as biological treasures. The arrival of rats on these islands could produce biological catastrophes but, contrary to common pessimistic assumptions, it is not inevitable that rats will arrive. Atkinson lists specific measures that reduce the risk. An immediate task is to secure the future of the remaining islands. □

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100 years ago

THE CONTINUITY OF THE GERM-PLASMA CONSIDERED AS THE BASIS OF A THEORY OF HEREDITY

THE present essay deals not with the entire subject of heredity, but with the fundamental question, How is it that a single cell of the body unites within itself the entire tendencies of inheritance of the whole organism? There are only two physiologically conceivable possibilities by which germ-cells endowed with such peculiar powers as we know them to possess can be produced. Either the substance of the parent germ-cell after passing through a cycle of changes required for the construction of a new individual possesses the capability of producing anew identical germ cells, or the germ-cells arise as far as their essential and characteristic substance is concerned, not at all out of the body of the individual, but direct from the parent germ-cell.

It is this latter view which Prof. Weismann holds to be correct, and maintains in the present essay, and which he terms the theory of the continuity of the Germ-plasma.

From *Nature* 33 154, 17 December, 1885.

Astrophysics

Bipolar wind from young stars

from James P. Emerson

THE molecular cloud L1551 contains the best example of a collimated bipolar high-velocity molecular outflow driven by a young stellar object. Now high-resolution ^{12}CO observations have directly demonstrated the postulated windswept cavity and shell in this cloud (Snell, R.L. & Schloerb, F.P. *Astrophys. J.* **295**, 490; 1985). The complex observed morphology and kinematics can be neatly explained by the combination of two effects. First, a collimated high-velocity flow from the young stellar object IRS5 (infrared source 5) sweeps out a bipolar cavity in the molecular cloud, and compresses much of the material into a dense thin shell surrounding the cavity; and second, the cavity expands uniformly into the surrounding cloud. The biggest puzzle of all, however, still remains — what causes the collimated bipolar wind from the young star in the first place?

A key problem of astrophysics is the formation and early evolutionary stages of stars. Interpretation of the observations of young high-mass stars is particularly complicated because they are usually associated with clusters of other nearby stars, surrounding ionized hydrogen regions, ionization fronts and shocks. Therefore, many researchers hoped to get a clearer picture of star formation from studying low-mass stars forming in dark clouds, because these are relatively isolated objects and not expected to be associated with the

complexities of ionized hydrogen regions.

Far from the anticipated simplicity, there has instead been mounting evidence that energetic jets and collimated outflows are commonly associated with young stars, even those of low mass. The energy input into the surrounding clouds from these bipolar winds could significantly affect the subsequent evolution of the cloud and its star-forming history. Some of these problems are analogous to those involved in understanding jets from galaxies, although jets from young stars are non-relativistic and do not involve such great energies (see Lada, C.J. *Ann. Rev. Astron. Astrophys.* **23**, 267; 1985).

When molecular-line observers first turned their telescopes towards the candidate young star IRS5, which is in the dark cloud L1551, they were surprised to find, in addition to the expected low-velocity molecular gas associated with the dark cloud, blueshifted and redshifted high-velocity gas located in oppositely directed bipolar lobes on the sky (see figure). The south-west lobe contains blueshifted gas and the north-east lobe redshifted gas. In the approaching lobe optical nebulosity can be seen because the flow is coming out of the dark cloud, but in the receding lobe no nebulosity can be seen because of the obscuring dust.

Although many examples of bipolar molecular outflows have now been found, L1551 remains prototypical as its proximity

and orientation make it especially favourable for detailed observational study. Optical studies of the south-west lobe have identified shock-excited knots of gas (termed Herbig-Haro objects) whose proper motions are radially outwards from IRS5. Optical and high-resolution radio observations have both shown ionized jets emanating from IRS5 parallel with the much larger-scale bipolar outflow and orthogonal to a dense rotating torus of high-density material seen in CS (carbon monosulphide) observations and suggested by infrared polarization studies. Shock-excited molecular hydrogen is

found at the edge of the flow, and some of the optical nebulosity is interpreted as reflected light from IRS5.

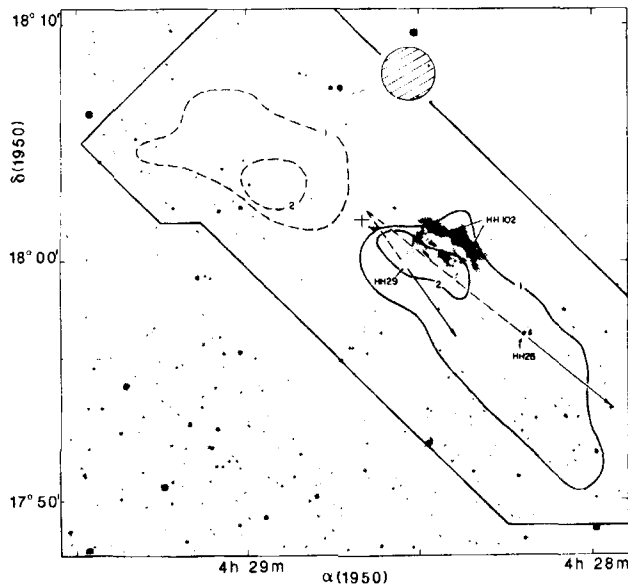
Snell and Schloerb have produced a complete ^{12}CO map of L1551 in the 2.6-mm line with the highest angular resolution (45") yet used on this flow, together with lunar occultation measurements of 7" resolution which clearly show the cavity and shell, and demonstrate that each lobe can be considered to be divided into two velocity components. The lower velocity component is in a thin shell at the periphery of the higher velocity component and contains a substantial fraction of the 0.9 solar masses of material in the outflow. The data and the overall flow shape can be neatly and consistently interpreted in terms of the collimated bipolar wind from IRS5 driving gas radially outwards at about 12 km s^{-1} , while the bubble thus created expands uniformly at a velocity of about 4 km s^{-1} . The mass now contained in the cavity and its surrounding compressed shell is close to that expected to be swept up if the cavity volume originally contained molecular gas at the ambient density of the undisturbed central parts of the cloud.

As Snell and Schloerb point out, although the mechanical luminosity of the wind is only a small fraction of the luminosity of the embedded star, the mechanism by which the energy of IRS5 is converted into momentum in the flow is as unknown in L1551 as it is in the other examples of this phenomenon.

It is widely believed that many young stellar objects, still evolving towards the main sequence, are surrounded by remnants of their parent cloud in the form of a rotating circumstellar torus or disk. It is crucial for theories of the formation of planetary systems and stars that the disks and their interaction with the stars and their immediate environment be understood. These disks are commonly assumed to be involved in the production of jets and bipolar outflows by an interaction between the young star and the surrounding torus or disk of material. It is thought that the interaction producing the jets is mediated by some combination of local density structure, magnetic fields, rotation and accretion of material, but theoretical models have failed to provide any widely accepted explanation.

Many of the current observations make use of techniques such as interferometry and polarimetry in the infrared and millimetre spectral regions in attempts to identify clearly these disks and to define their properties and how they relate to the jets (for example, Beckwith, S. *et al. Astrophys. J.* **287**, 793; 1984 for HL Tau, which also lies in L1551), but it seems more probable that a new theoretical model is required to explain how the collimated jets from young stars are driven. □

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An earlier lower resolution ^{12}CO contour map of the L1551 outflow (from Snell *et al. Astrophys. J.* **239**, L17; 1980) in the broad-velocity components, superimposed on an optical photograph of the L1551 region. Dashed contours, redshifted molecular gas; solid contours, blueshifted gas. Also shown are the direction of the proper motions of the two compact Herbig-Haro objects, HH28 and HH29; tracing their motion backwards suggests a common origin at IRS5 (cross). α , Right ascension; δ , declination.

Drosophila development

Homing in on homoeo boxes

from Geoffrey North

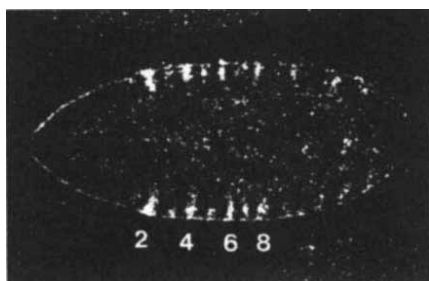
SINCE its discovery^{1,2} as a short repeated sequence within the homoeotic gene complexes of the fruitfly *Drosophila melanogaster*, research on the 'homoeo box' has taken three more or less independent directions. One has been the pursuit of evidence that the presence of homoeo boxes in the genomes of other species really does signify that they have equivalents of the fruitfly's homoeotic genes. The state of this particular art was discussed recently in these columns³. A second has been cataloguing of all *Drosophila* genes that have homoeo boxes, the newest member of the club being *caudal*⁴, which is the first example that is 'maternally expressed' and so presumably concerned with the very earliest events of development. And the third has been the attempt to ascribe a function to the homoeo box. This has now borne fruit, with the evidence reported by C. Desplan, J. Theis and P.H. O'Farrell on page 630 of this issue⁵ that the protein domain encoded by the homoeo box of the *engrailed* gene of *Drosophila* has sequence-specific DNA-binding activity.

But first some recent history. Shortly after their discovery^{1,2} as repetitive sequences of both the bithorax and Antennapedia gene complexes of *Drosophila*, homoeo boxes were found to encode roughly 60 highly-conserved amino acids, which would form a domain within the protein products of each of the genes that constitute these complexes. Although most of these genes are strictly homoeotic in that their mutations cause clean transformations of one body-part into another, one of the first homoeo boxes to be sequenced belongs to a rather different kind of gene. This is *fushi tarazu*, the odd-man out in the Antennapedia complex, which falls into the 'pair-rule' class of segmentation genes: in *fushi tarazu* mutant embryos, equivalent regions of every other segment are deleted.

The diversity of genes that contain a homoeo box was increased still further with *engrailed*^{6,7} — the first to be found outside the bithorax and Antennapedia complexes. *Engrailed* has some properties in common with the classical homoeotic genes, but others that are more reminiscent of segmentation genes such as *fushi tarazu*. Thus it seems to specify the posterior compartment of every segment of the developing fruitfly, for if clones of mutant cells lacking wild-type *engrailed* activity are created in a posterior compartment they develop as though they were in the equivalent region of the anterior compartment of the same segment. But in *engrailed* mutant embryos, unlike classical homoeotic mutants, the pattern of seg-

mentation is disrupted. In fact, the effect resembles that of a pair-rule mutation, which may relate to the observation that the developing pattern of *engrailed* expression, which in its final form is a series of stripes corresponding to the eventual pattern or morphological segmentation, goes through an intermediate stage with a clear-cut two-segment periodicity^{8,9} (see figure).

Thus, although all of the *Drosophila* homoeo-box-containing genes, including *caudal*, seem to have interesting roles in



Expression of *engrailed* with a two-segment periodicity (from ref. 8).

development, at the macroscopic level they seem to have a variety of types of function. But the implication of their shared homoeo-box domain is that at the molecular level the products of these genes work in basically similar ways. A clue as to what this common activity might be has come from the homology of the *Drosophila* homoeo boxes to regions of the yeast *MAT a* and *MAT a2* genes^{10,11}, which are known to regulate sets of genes that determine the mating type of haploid yeast cells. The protein product of the *MAT a2* gene has recently been shown to bind specifically to a sequence located upstream of the genes that it regulates¹².

It is against this background that Desplan *et al.* have tried to determine whether, as widely predicted, the *Drosophila* homoeo boxes also encode DNA-binding domains. Following a similar approach to that of Johnson and Herskowitz in their study of the yeast *a2* protein, Desplan *et al.* have constructed fusion proteins in which different regions of the *engrailed* protein product are tagged on to the end of the enzyme β -galactosidase. Expressed from hybrid genes introduced into the bacterium *Escherichia coli*, high enough levels of these fusion proteins can be obtained for biochemical studies.

Compared with Johnson and Herskowitz, however, Desplan *et al.* were at a disadvantage, for they had no prior indication of the sequences to which the *engrailed* protein might bind. So at first they simply tested whether their fusion proteins would bind non-specifically to frag-

ments of the bacteriophage lambda genome. Indeed, they found that those fusion proteins that contained the *engrailed* homoeo-box domain did have DNA-binding activity. But was this simply due to a non-specific interaction between acidic DNA fragments and the basic protein molecules? It seems not, for under more stringent conditions it was revealed that some of the lambda fragments are bound much more avidly than others. This is a clear sign that the DNA-binding activity of the fusion proteins is of the legitimate, sequence-specific kind. Presumably it is just by chance that some lambda fragments fulfill some of the sequence requirements for binding the *engrailed* protein, but it could well be that the fusion proteins are rather less choosy *in vitro* than *in vivo* (where their specificity might well be increased by interactions with other proteins).

Finding the functionally significant sequences of the *Drosophila* genomes to which the *engrailed* protein is presumed to bind is a more difficult problem, which Desplan *et al.* have approached by following the increasing genetic evidence that genes such as *engrailed* are involved in mutual regulatory interactions. They have therefore looked for and found binding sites for the *engrailed* protein upstream of the *engrailed* gene itself — but, as the authors themselves stress, this highly intriguing result must be interpreted with caution, for the affinity of the protein for these sites is not significantly different from that for its favourite fragments of the lambda genome. The binding sites near the *engrailed* gene are conserved between two different *Drosophila* species, however, which implies some functional significance. If it turns out that they are not true binding sites for the *engrailed* protein perhaps they are binding sites for some other protein that contains the homoeo-box domain. An example would be the product of the *fushi tarazu* gene, which recent genetic evidence suggests is involved in establishing the pattern of *engrailed* expression during the early development of *Drosophila* embryos (Phil Ingham and Ken Howard, personal communication). □

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Geoffrey North is Deputy Biology Editor of *Nature*.

Quasiperiodic order in dissipative systems

SIR—The concept of quasicrystalline order¹ in relation to experimental observations²⁻⁴ in Al_3Mn suggests that quasiperiodic structures are also likely to appear in driven systems undergoing patterning instabilities. (Classical examples are given by convective instabilities in fluids or Turing instabilities in chemical systems, for example, ref. 5). Despite the complexity of the dynamics, such symmetry-breaking instabilities may be described near threshold by a kinetic equation for the unstable or critical modes which play the role of an order parameter. They are related for example to the velocity field in hydrodynamical systems or to the concentration of active species in chemical systems^{6,7}. The stabilities of the patterns may be ordered in decreasing values of the associated Lyapunov functional which plays the role of the Ginzburg-Landau-Brazovskii potential in equilibrium situations.

Near threshold, structures built on wavevectors of critical wavelength may be stable and when the Lyapunov functional is limited to its fourth order invariant a situation very similar to the Landau treatment of crystallization is recovered⁸. Among the possible patterns one finds, for example, layered structures corresponding to modulations in one direction only, bimodal patterns and structures with triangular or cubic symmetry corresponding to combinations of critical modes with wavevectors forming equilateral triangles⁹.

However, we have to keep in mind that the kinetic equation for the order parameter-like variables is obtained via the adiabatic elimination of stable noncritical modes which are driven by the critical ones. Hence their contribution may become important for increasing values of the bifurcation parameter. As a result, the intensity of the coupling between three modes is renormalized and, furthermore, higher order nonlinearities are generated by the driven modes as in the multicomponent Landau theory⁴. The first nonlinear coupling induced by this effect in the kinetic equation involves four critical modes corresponding to a fifth order invariant in the Lyapunov functional¹. This term favours the formation of structures built on wavevectors forming an equilateral pentagon which in two-dimensional systems has the symmetry of a Penrose lattice.

The emergence of high order nonlinearities in the dynamics of the critical modes favouring quasiperiodic patterns is a general feature of this description of systems undergoing symmetry-breaking instabilities^{6,7}. Hence, according to the intensities of the nonlinear couplings which are system dependent, quasiperiodic structures could become the stablest ones

in some range of the parameters. Having a dominant growth rate versus layered structures they should also appear after sudden increases of the control parameter.

In various convective instabilities, the increase of the constraint beyond threshold leads to the nucleation of defects and ultimately to the destruction of the translational order⁹. It was suggested that this effect could be related to a dislocation induced melting similar to what happens in two-dimensional solids^{7,10}. From the present analysis, however, one may also expect the appearance of quasiperiodic structures well beyond threshold. It would thus be interesting to perform diffraction experiments for such systems after slow and sudden increases of the bifurcation parameter in the region where translational order is lacking to test whether the disorganization is related to a dislocation-induced melting or to the transition from periodic to quasiperiodic structures.

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The cosmic connection of catastrophism?

SIR—The remarks by Weissman¹ on the review by Maddox² require some additional comment. This relates to a possible extraterrestrial cause of terrestrial catastrophism and, in particular, to the origin of the suggestion that this might be associated with the Sun's motion in the Galaxy.

As previously pointed out in these pages³ pulsations are not a new idea and were suggested more than half-a-century ago⁴. Later, in 1947, Umbgrove⁵ reasoned that the Earth had a periodicity of about 250 Myr and drew attention to the similarity between this and the galactic day. Although Umbgrove was probably the first to make such a suggestion, the validity of much of his evidence can certainly now be questioned⁶.

Probably the first experimental measurements relevant to this topic were carried out in the early 1960s⁷ and the re-

sults were published in this journal in 1971⁸. These measurements indicated that the times of emplacement (in Myr) of mantle-derived carbonatites (for at least the last 1.7 Gyr) could be approximated very closely by the relationship $T_n = 233n - 102$, with n integral. The similarity of this period with that for galactic rotation was then noted. The significance of the data set used in deriving this conclusion has since been tested⁹. From other evidence Innanen *et al.*¹⁰ have subsequently proposed a galactic model from which they derive an identical numerical value of 233 Myr for the length of the galactic year.

In 1971, plate tectonics was in its infancy and episodicity and global synchronicity were rather unfashionable concepts in geology. Over the years, this data set has therefore been continually expanded and improved¹¹ and incorporated into a hypothesis of Earth behaviour¹², compatible with plate tectonics, which nonetheless preserves this fundamental periodicity. This hypothesis is testable by carrying out more accurate age measurements on carbonatites.

One striking feature of the time distribution is the near coincidence of times T_1 , T_2 and T_3 with the boundaries of geological periods in Phanerozoic time¹³. It is therefore tempting to extrapolate backwards ($n \geq 4$) for a Precambrian timescale. Another corollary of this hypothesis is that, as a large number of carbonatites exist with ages around 65 Myr (see, for example, refs 12, 14), an atmospheric spike of carbonatite-derived CO_2 may have influenced events near the Cretaceous-Tertiary¹⁵ boundary. This temporal coincidence can again be tested by accurate geochronological measurements. Whether, in addition, widespread carbonatite emplacement can explain the well-documented geochemical anomalies associated with this boundary is currently being examined by analysing rocks from (dated) carbonatite complexes which were emplaced across the boundary.

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How did we get to here from there?

Richard Wilson

Nuclear America: Military and Civilian Nuclear Power in the United States, 1940–1980.

By Gerard H. Clarfield and William M. Wiecek.

Harper & Row: 1985. Pp.518. \$19.95, £20.95.

Controlling the Atom: The Beginnings of Nuclear Regulation, 1946–1962.

By George T. Mazuzan and J. Samuel Walker.

University of California Press: 1985. Pp.530. \$28.95, £24.50.

Radiant Science, Dark Politics: A Memoir of the Nuclear Age. By Martin D. Kamen.

University of California Press: 1985. Pp.348. \$19.95, £16.95.

ON August 6, 1945, I was at a camp at Titchfield, Hampshire, England, when I heard the news that an atomic bomb had been dropped on Japan. A year later I began my studies in physics for the DPhil. at Oxford. There, in the Clarendon Laboratory, I met some of those who participated in the atomic bomb programme, among them "Jim" Tuck, who had gone to Los Alamos as an explosives expert and returned to build a betatron, and Professor Franz E. Simon, who with others had worked on gaseous diffusion in the rooms below the undergraduate optics laboratory.

In that winter of 1946–1947, the severe weather prevented transport of coal from the mines to power stations for nearly six weeks; stocks were low and Britain had its first electricity rationing. As physicists we realized that this short-term problem was merely an indicator of a future long-term one, and at tea times discussed the solutions to the "energy crisis". On the one hand, we could burn coal more efficiently, and with less environmental disadvantages; gasify it underground and use the gas in a thermodynamically efficient fuel cell. And then there was nuclear power.

Since 1939 it had been clear that nuclear fission might be used either for power or for bombs. The Second World War had given work on bombs priority, but after 1945 scientists returned to their first love, nuclear electric power. The problems of proliferation of weapons among countries were clear. We concluded that within 30 years (by 1976) 30 countries could and would have bombs. But we were pessimistic; most countries that could do so have declined to make them. In another way, we were too optimistic. It never occurred to us that two countries, the United States and the Soviet Union, would possess tens of thousands each.

We were over-optimistic, too, about the speed of nuclear power development; instead of the five years expected, it took ten years to produce the first power plant (in Britain in 1956) and 25 additional years for much market penetration. We were at the same time pessimistic about the need for power. We had not anticipated that oil from the Arabian (at that time Persian) Gulf would be available at such an incredibly low cost.

We were very aware of the environmental advantages of nuclear power. Many of us had lived in London in the days of the bad fogs, and it must be remembered that the fog of early December 1952, which caused 4,000 "excess deaths", was far from the worst; it was merely the first about which there are good health statistics. At the same time we were all well aware that nuclear power is only conditionally safe — conditional on the technology being designed and operated well.

What went wrong with our projections? Why, after a promising start 30 years ago, is the nuclear power industry in such trouble in the United States? Why is the number of bombs increasing, apparently without limit? In other words, how did we get to here from there?

With all the many books about nuclear electric power that have been written, I have found none that discusses this all-important question. To illustrate this surprising omission of the world's scribblers, I discuss three of the recent books about the nucleus and man's attempts to tame it.

None of the three books gives us a clear answer. But the book by Clarfield and Wiecek, historians from the University of Missouri, strongly implies that we should abandon nuclear technology completely. This is a dangerous conclusion and unfortunately typical. Errors of fact in the book show that little reliance can be placed on the authors' justification of their case.

Clarfield and Wiecek consistently imply that military and civilian uses of nuclear

energy are inseparable and that we cannot stop one and not the other. This is a common view nowadays in the United States. But I regard as more tenable the proposition that military and civilian uses of nuclear power *are* separable and that world peace may depend on our ability to make the distinction clear. American anti-nuclear activists often damn bombs and power reactors equally; but the record shows that although activists have prevented many reactors from being built, it is doubtful whether they have reduced the military stockpiles by a single bomb. By pretending the two targets, bombs and power stations, to be equally dangerous, they have allowed themselves to be diverted from the military target and attacked the easier, more vulnerable, civilian target. Worse still, they have separated themselves from their natural allies in the scientific community and weakened the peace movement.

The layout of the book itself belies Clarfield and Wiecek's thesis. While the prologue and Chapter 1 are "mixed" chapters, about nuclear fission and its discovery, later chapters are *either* about bombs *or* about electric power reactors; not both. Only in the polemical epilogue do the two come together again.

In their discussion of nuclear safety, Clarfield and Wiecek tell their story from the outside looking in and would seem to be objective. But their technical discussions are simply wrong. Referring, as have other authors, to the Emergency Core Cooling Hearing of 1972–1973, they make the incorrect statement that "the AEC [Atomic Energy Commission] remained unmoved by these disclosures, and made no significant changes in its safety regulations". On the contrary, the AEC made enough changes to make the uncertainties disclosed in the hearing moot. The authors ignore the fact that tests of the emergency core cooling systems in 1978 showed that even the original criteria were conservative and had a margin of error.

Clarfield and Wiecek state that "Rasmussen actually had little to do with the substance" of the Reactor Safety Study of

IMAGE
UNAVAILABLE
FOR
COPYRIGHT
REASONS

Promising start: the Queen opens Calder Hall nuclear power station, October 17, 1956.

1975 of which he was in charge. This seems very unlikely; this year, the US Department of Energy awarded Rasmussen the Fermi Prize for his work. It is also untrue that "the Rasmussen report did not stand up well to criticism". On the contrary, the methodology is now almost universally used, and even critics rarely discuss reactor safety in any other framework. A recent study group of the American Physical Society gave it greater praise ten years later; it was found unnecessary to comment upon it specifically, but the results were used as a baseline with which to compare later calculations.

The several pages about the accident at Three Mile Island are particularly sloppy. The authors state that "the problems at TMI began when pumps forcing cooling water through the primary system tripped". It was the secondary, not the primary, pumps that tripped. The primary pumps were manually, and foolishly, shut down two and three-quarter hours later when they began to cavitate. The confusion between the primary and secondary systems is evident later in the same sentence when again they say that "temperatures began to rise in the core because the coolant was no longer flowing", clearly referring to the primary system, and then in the next sentence state that "this was a few seconds after the start of the accident". It was over two hours after the start of the accident that the primary system flow stopped and core heating really began.

More serious for a couple of historians is the uncritical mention of the worry of Dr Bridenbaugh about "the obvious use to which the government of India was putting its Tarapur reactor, the first 'peaceful' application of nuclear power in the Third World. The Indian government was reprocessing plutonium out of the spent fuel to build a bomb . . ."; this statement is almost certainly wrong. There is no evidence for it, and a lot of evidence against it. Tarapur and its fuel are under international safeguards and inspection. Both the Indian Government and the International Atomic Energy Agency say that the fuel has been stored, and the United States has a right in the agreement to take it back, unprocessed. More importantly, the reprocessed fuel would yield plutonium which has too much of the isotope ^{240}Pu , thereby making it unsuited for any but an unreliable bomb, and in India has other reactors of its own manufacture, not under safeguards, which are more suited to producing high quality, bomb grade, plutonium. Why should India break agreements and treaties, and risk being caught by an inspection, to get an inferior product?

In the second book under review, *Controlling the Atom*, I had hoped to find a clear description of the present regulations, how they led to the current situation, how France and Japan have avoided the problems, and how we might change



In defence of Martin Kamen: a cartoon in the *St Louis Star-Times*, September 4, 1948.

the situation by changing the regulations. The authors, Mazuzan and Walker, are historians at the Nuclear Regulatory Commission in Washington, and should be well equipped to do so. I do find a clear description of the present regulatory process in the United States although no comparison with its equivalents overseas. The problem arises because the book is strictly limited to the period 1946 to 1962, so that the failure of the industry in the past ten years is not even mentioned.

Nonetheless, this book makes interesting reading. The quotations make it clear that those who set up the regulatory procedures were as aware of the general safety problems as we are today. The book reminds us of the Brookhaven Study on catastrophic accidents which was presented to the Commission and released to Congress and the public on March 15, 1957, labelled WASH-740, and included the numerical estimates that some scientists gave to describe their "feeling" for the probability of accidents. Three estimates were given. Under the assumption of 100 operating reactors, and that 3,000 deaths would result from a major release outside the containment, one of them gave an individual risk of 1/50,000,000 per year of a person being killed by a reactor accident.

Eighteen years later, on February 21, 1985, I presented to the Nuclear Regulatory Commission a report prepared by a Study Group of the American Physical Society on "Radiological Consequences of Severe Nuclear Reactor Accidents". We concluded that an accident violating the containment is now much less probable than at the time of WASH-740 and although the reactors are bigger than anticipated in 1957, the consequences are likely to be smaller and 3,000 deaths would be very unlikely.

Thus it seems that the Brookhaven scientists were overly pessimistic. Although most of the American public were not interested and did not notice WASH-740 when it was released, the often-repeated

accusation that facts were withheld was, in general, untrue.

The book by Kamen, a distinguished chemist and co-discoverer of carbon-14, is a personal memoir. Kamen worked in the Radiation Laboratory at Berkeley from 1935 to 1944, and his book describes his early excitement as a scientist and his dismay at being dismissed from the Manhattan project in 1944.

It appeared later that his dismissal was not due to specific charges of disloyalty, but because he was a gregarious man with a wide circle of friends, some of whom were under suspicion. Kamen became more of a security risk than his usefulness to the project justified. Nonetheless he was denounced as "that atom spy" by the *Chicago Tribune* and *Washington Times-Herald*, but he won his libel suit against the one and the other settled out of court. His passport was withheld for seven years by Mrs Shipley, chief of the Passport Division of the US Department of State. The Department of Justice later stipulated that this was illegal. All this makes interesting reading and tells of how Americans can be misled by publicity hounds. But it also tells us another story. Bombs and power plants are only two of many uses of nuclear fission.

Kamen describes little of the scientific and technical work of the Manhattan Project in this book. It is clear that neither bombs nor electric power interest him. His scientific interests were other applications of nuclear science — first in isotopic tracers then cytochrome chemistry, biological oxidation, photosynthesis and protein structure. Radioactive isotopes in research and medical work are a third important use of nuclear fission in modern life. Kamen describes his excitement when he realized that some radioactive isotopes made for his work during the war were so radioactive that they must have been made by a nuclear reactor, rather than by a radium beryllium source. It was the medical applications of such work that earned Dr Rosalyn Yalow her Nobel Prize in medicine.

Small reactors have been built in a number of countries under the Atoms for Peace programme. It was a research reactor *not* a nuclear power reactor that was used by India to produce plutonium for a nuclear explosion. If we want to ban all nuclear technology in order to stop nuclear bombs, we would have to close down these research and isotope-producing reactors and thereby put an end to Kamen's and Yalow's research. The solution most of the world accepts is to allow both types of reactor to exist provided that there is international inspection under the Non-Proliferation Treaty. This is better than the chaos Clarfield and Wiecek would lead us to. □

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Searching for their selves

Stuart Sutherland

The Man Who Mistook his Wife for a Hat. By Oliver Sacks. Duckworth: 1985. Pp.233. £9.95. To be published in the United States in January by Summit, \$15.95.

In his latest book, the author of *Awakenings* reports many curious happenings. Perhaps the most surprising is his revelation that one can be a practising neurologist without having read the literature on visual agnosia and without knowing that retrograde amnesia is common in Kors-



Oliver Sacks: unusual neurologist.

koff's psychosis, and indeed after medial temporal lobe damage in general. Oliver Sacks asserts that he only acquired the knowledge in question after having encountered such cases himself: it is of course possible, judging from *The Man Who Mistook His Wife for a Hat*, that he had been too busy reading Wittgenstein and Nietzsche to be bothered with the literature of his profession, but one fears he may be having the reader on, a suspicion that the remainder of his book does little to allay.

Dr Sacks recounts with verve and clarity some score of neurological case-histories, all of which make excellent short stories. He describes a wide range of symptoms, ranging from Tourette's syndrome, which is marked by tics, grimaces, curses, and "an odd elfish humour . . . and outlandish kinds of play", to those of an idiot savant. His cases have only two unusual features. First, they seem to present symptoms in an exceptionally pure form and do not exhibit the messy confounding of different syndromes so common in such patients. Second, they were treated by a very unusual neurologist, Dr Sacks himself.

He is unusual in that no matter how

devastating the incapacity caused by his patient's brain damage, he always seems able to locate, usually at the first interview, an area in which the patients' "self" is still intact and which they can be encouraged to develop. This may relieve their misery — where they are miserable, since many neurological patients have no understanding of their condition and may even be unnaturally cheerful. Moreover, the development of an intact interest or skill may, according to Sacks, help to alleviate patients' symptoms. He points, for example, to a mentally retarded girl with extremely clumsy body movements, whom he helped find herself through acting: when she was on the stage all her clumsiness disappeared.

Although Sacks's grasp of brain science seems at times a little shaky (for example, Wilder Penfield did not show "beyond doubt" that epileptic transports are direct

memories of the past rather than fantasies), his message is important and compassionate. He writes, "Our tests are ridiculously inadequate. They only show us defects . . . they do not show us a being conducting itself spontaneously in its own natural way". If we are to believe him, the discovery and promotion of the spontaneous self is particularly important in neurology, but it should surely play a role in all medicine.

The book deserves to be widely read whether for its message, or as an easy introduction to neurological symptoms, or simply as a collection of moving tales. The reader should, however, bring to it a little scepticism, for outside Sacks's clinic, things do not always fall out quite so pat. □

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Different biology

Alan Bittles

Human Sexual Dimorphism. Edited by J. Ghesquiere, R.D. Martin and F. Newcombe. Taylor & Francis: 1985. Pp.375. £24, \$53.

ALTHOUGH, perhaps inevitably, there is considerable variation in the content and quality of the 18 contributions in a volume of this nature, based on a joint symposium organized by the Society for the Study of Human Biology and the European Anthropological Association, the editors have assembled an informative and very readable collection of papers on this important biological topic. From a purely semantic viewpoint, use of the term sexual dimorphism in the book is often somewhat inappropriate as the parameters can display considerable overlap between the sexes and show greater intra- than inter-group differences. The authors in general have adopted a fairly flexible, if occasionally rather arbitrary, definition for the expression of dimorphism in a species, and for this reason some of the conclusions may not necessarily be definitive.

Allometric analysis is used by a number of contributors in an attempt to unravel the circularity apparent in theories constructed to explain the origins of dimorphism. By scaling brain size to body size in simian primates it is apparent that dimorphism has evolved through reduced body size in females rather than increased male body size. Further, similar studies across a wide range of mammals have shown that brain weight is primarily associated with age at sexual maturity, rather than with lifespan as has been commonly supposed. Possible mechanisms governing embryonic sexual differentiation via H-Y antigen secreted by the Sertoli cells, and sex-differentiated behaviour

patterns are clearly presented. Given the difficulties inherent in obtaining experimental evidence from human studies, the elegant demonstration in zebra finches of a hormone-influenced dichotomous choice between male and female patterns at single neurone level, and the subsequent translation of the degree of brain masculinization into a behavioural continuum, may be of considerable significance.

While evidence from our hominid ancestors and the australopithecines relating to sexual dimorphism is both limited and literally fragmentary, the existence of dimorphic patterns in *Homo sapiens* is well established. As expected from the title, human sexual dimorphism is thoroughly assessed in a series of six papers on anthropometric, physiological and behavioural findings from a variety of extant populations in Europe, Africa and Asia. The possibility that apparent dimorphic differences may have arisen as a result of acculturation, rather than being representative of man's basal state, is illustrated in an extensive project conducted on Aboriginal hunter-gathers from Arnhem Land in northern Australia. Somewhat surprisingly, in this population the age-associated rise in blood pressure is a female, not a male, phenomenon which calls into question many of the assumptions made regarding the basic biology of our species, derived only from groups living in the developed countries.

Despite the less than perfect reproduction of the camera-ready format, overall this is an impressive publication which should find a ready audience across a wide range of biologists, behavioural scientists and clinicians. It can be recommended both as a source text and an interesting volume in its own right. □

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Feeding and flocking

C.M. Lessells

Gulls and Plovers: The Ecology and Behaviour of Mixed-Species Feeding Groups. By C.J. Barnard and D.B.A. Thompson. *Croom Helm/Columbia University Press: 1985. Pp.302. £25, \$30.*

THE idea that natural selection can act on an animal's behaviour, as well as its more tangible characteristics such as morphology, is well established. Behaviour is not now merely described and measured but is interpreted in terms of the selection pressures responsible for its evolution and maintenance. Barnard and Thompson follow in this tradition with their study of lapwings, golden plovers and black-headed gulls feeding on worms in agricultural pasture.

At first sight, winter feeding and flocking behaviour are really rather mundane, but this book reveals the extent of the interdependence of feeding and flocking strategies within and between the three species: foraging choices of individuals determine the size and location of feeding flocks, while the composition of flocks in turn affects feeding rates and hence the best foraging strategies. To add to this, the black-headed gulls make their living by stealing worms from the plovers, while the plovers may exploit the gulls for early

warning of the approach of predators.

Clearly Barnard and Thompson have set themselves quite a task in unravelling the complexities of this system. Their quantitative approach to the problem relies on a wealth of data, so much so that the book is rather hard going. Ultimately the success of the book must be judged by whether they are able to cut a path through the jungle of tables and graphs.

As in many other studies, the difficulties firstly of assessing costs and benefits of alternative actions, and secondly of reconciling the different units (such as calories and probability of predation) of costs and benefits, have made quantitative predictions rather thin on the ground. Precise quantitative predictions, however, can be made about the choice of food items by feeding plovers and thieving gulls. The "optimal diet model" provides a well-worked solution to both problems, but here it has been mangled almost beyond

recognition. Profitability, encounter rate, availability and prey types are all incorrectly defined; for instance equation (8.2) is akin to saying that the overall effect of driving for a while at 20 mph and a while at 30 mph is driving at 50 mph. Consequently the line of argument is weakest where it should be strongest. Similarly, innovative thinking elsewhere in the book is marred by inadequate explanation, confused terminology or misused statistics.

Despite this there is much interesting new detail in the book and some new ways of looking at the interrelationships of flocking and foraging strategies. Barnard and Thompson would have contributed more, however, if in their genuine attempt to push back the frontiers of darkness they had not extinguished the lights behind them. □

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Isolated objectives

C.J. Marshall

Molecular Cell Genetics. Edited by Michael M. Gottesman. Wiley: 1985. Pp.931. £82.30, \$79.95.

MUCH of the work on the genetics of cultured cells has made use of Chinese hamster cells, especially the CHO cell line originated by T.T. Puck, the founder of cell genetics. The importance of these cells is reflected in the emphasis of this book and it is worth noting that, as Yerganian describes, the use of Chinese hamsters is not without heroic and even tragic episodes — during the Revolution a Chinese research worker was imprisoned for his involvement in the shipment of animals to the United States.

The reasons why the CHO cell line has been used so extensively are partly historical, but perhaps more important is the comparative ease with which recessive phenotypes can be isolated in this cell line. Siminovitch, in 1976, proposed that recessive mutations could be isolated in CHO cells because some loci were hemizygous as a result of chromosome rearrangements.

Subsequent studies described by Siminovitch and others have confirmed and extended these ideas. For some autosomal loci, CHO does appear to have only one copy of the genes; however, other loci are diploid and there is increasing evidence that one locus can be inactivated or deleted by a high frequency event (10^{-3} or greater) to uncover recessive mutations. Such events may have profound implications for our understanding of alterations in gene activity that lead to neoplastic transformation.

In *Molecular Cell Genetics*, the editor lists over 100 mutant phenotypes in

Chinese hamster cells which include nutritional auxotrophic requirements, mutations in mitochondria, RNA polymerases, protein synthesis, DNA repair, lysosomes, membrane proteins, glycosylases, protein kinases and many others.

As is to be expected of a multi-authored book, the chapters in *Molecular Cell Genetics* are of variable quality. Some authors have written overviews of an area, often drawing very heavily on their own studies; others have provided both a review and useful hints for particular procedures. In most cases detailed experimental protocols are not provided, so this is not a laboratory manual but rather a source book which would enable one to find the appropriate protocol. Over half the chapters are concerned with particular mutant systems, while others describe features of Chinese hamster cells and methods for cell genetics such as cell fusion, gene transfer, and cloning and expression of cDNAs. Much of the work still appears to be at the stage of characterizing mutants rather than using them to dissect a particular system or probe the structural function of a protein.

For much of the book I felt that a great deal was expected of the next development: the isolation of the relevant genes. For some loci this has already been achieved; however, there is still much to be done before many of the mutant phenotypes can be exploited for what the editor terms "molecular cell genetics": the synthesis of somatic cell genetics with molecular biology.

For those working in this area, or thinking of entering it, this book will be a valuable source of information. They might like to bear in mind, however, that there is more to cell genetics than *Cricetulus griseus*. □

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QUALITATIVE OBSERVATIONS ON RECENT FORAMINIFERAL TESTS . . .

WITH EMPHASIS ON THE EASTERN PACIFIC PARTS 1-111, 1977 — WITH EMPHASIS ON THE ATLANTIC, PART IV, 1981

by Irene A. McCulloch, PhD

This monographic work, in preparation for more than 30 years, consists of three parts on the Pacific, totalling more than 1000 pages with 200 folio-sized plates and covering some 360 genera and 2,300 species. Part IV on the Atlantic consists of 361 pages with 72 folio-sized plates covering more than 700 taxa. The folio-sized volumes are on 50% rag paper, bound in durable dark red Buksyn with gold stamped covers.

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Localizing active sites in zeolitic catalysts: neutron powder profile analysis and computer simulation of deuteropyridine bound to gallozeolite-L

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The location of a guest organic base within a model zeolitic catalyst can be quantitatively determined from an analysis of the neutron powder diffraction profile. The calculated position and orientation of the guest species within the channel agree well with the experimental values.

THE analogy between the mode of action of enzyme catalysts and zeolites is more than superficial. In each case, folds or cavities in the catalysts impose shape-selectivity that governs the 'choice' of reactant species, and the molecular complementarity of the microenvironment of the active site facilitates ensuing chemical conversion. Paradoxically, progress in characterizing the nature of active sites in enzymes (and, therefore, in engineering new proteins^{1,2}) is more advanced than that in characterizing the precise nature of the active site in zeolites. Notwithstanding their greater molecular complexity, enzymes can be made to yield good-quality single crystals which are then amenable to X-ray crystallographic analyses. Moreover, since the advent of genetic engineering, theories of enzyme catalysis in general, and views about active sites in particular, can be tested by delicate alteration of the enzyme structure. With zeolites, however, the situation is rather different. Although rapid progress has recently been made in the synthesis of new zeolitic catalysts³⁻⁵, it is very difficult to prepare them as defect-free, single crystals of a size and quality that permits X-ray analysis of the reactant-catalyst complex. Consequently, it has not yet been possible to emulate, using aluminosilicate zeolitic catalysts, work such as that done to locate fructose-6-phosphate and ATP at the active site of fructophosphokinase^{1,2}.

Alternative approaches to the elucidation of the structure of zeolitic catalysts have, therefore, been evolved⁶⁻²². These include application of such techniques as high-resolution electron microscopy⁶⁻¹¹, atom-atom calculations^{12,13}, magic-angle spinning NMR (MAS-NMR)¹⁴⁻¹⁷, and neutron¹⁸⁻²² and X-ray^{23,24} powder profile analysis. Of these techniques, the neutron profile method yields quantitative atomic structural information; it has been used to determine the location of the detachable proton in the (Brønsted)-active site in La^{3+} ion-exchanged zeolite-Y catalyst²¹, and also the siting of guest species such as Xe (which serves as a model for methane) in zeolite- ρ ²², and CO in zeolite-A²³. The precise location of sorbed benzene in zeolite-Y has also been determined by neutron powder profile analysis²⁶. Here we identify in atomic detail the siting of pyridine (which, together with other nitrogenous bases such as 4-methylquinoline²⁷, is used to block active sites) in the channels of a synthetic zeolite-L, and compare our results with a computer simulation based on atom-atom potentials.

Neutron powder diffraction analysis

The particular catalyst preparation used is a potassium salt of a gallosilicate-L, in which Ga replaces Al in the well-known structure (Fig. 1) of the anion framework²⁸. Chemical analysis gave a unit cell composition of $\text{K}_{10.3}\text{Ga}_{10.3}\text{Si}_{25.7}\text{O}_{72}$ for the dehy-

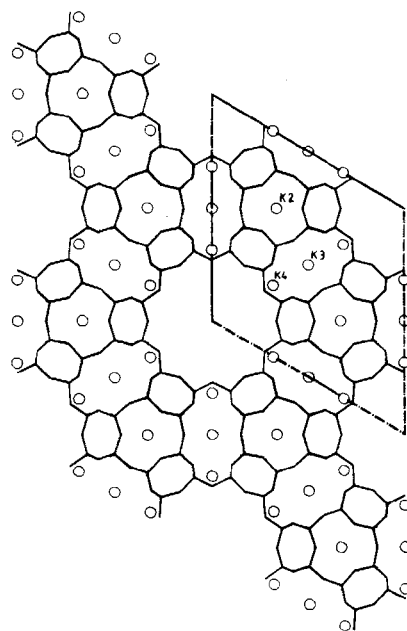


Fig. 1 A schematic representation of the structure of potassium zeolite-L, viewed down [001]. The aluminosilicate framework is indicated by lines and the cation positions as small circles. The large channel runs parallel to [001].

drated sample. Samples were characterized by X-ray diffraction and by MAS-NMR. High-quality, microcrystalline samples of the potassium salt (henceforth designated KGaL) yielded good neutron diffraction (powder) patterns, both in the dehydrated state and after incorporation into the dehydrated solid of ~1.2 molecules of perdeuteropyridine per unit cell. Data were recorded at 4 K at the Institut Laue-Langevin (ILL), Grenoble, using the high-resolution powder diffractometer, DIA.

There were some 70 discernible peaks in the 2θ -range with a neutron wavelength of 1.909 Å. Refinement of the structure, using Rietveld profile analysis^{18,19}, was done as follows. First, the starting coordinates of a previous refinement of KAIL²⁸ were assumed for the KGaL. Scattering lengths were taken from Bacon²⁹: $b_{\text{O}} = 0.575$, $b_{\text{N}} = 0.940$, $b_{\text{C}} = 0.663$, $b_{\text{K}} = 0.37$, $b_{\text{D}} = 0.67$, all in units of 10^{-14} m. The scattering of the tetrahedral sites was calculated from a weighted average of the scattering lengths of silicon ($b_{\text{Si}} = 0.415$) and gallium ($b_{\text{Ga}} = 0.720$) and the occupancies refined. Refinement progressed smoothly; the occupancy factors of the two distinct tetrahedral sites were

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Table 1 Atomic coordinates, isotropic thermal parameters and occupancies (number per unit cell, *N*) for KGaL (A) and KGaL + pyridine (B)

Atom	x	y	z	B	n
A					
T-1	0.0933(4)	0.3569(4)	0.5000	0.1(1)	12
T-2	0.1672(4)	0.5001(4)	0.2128(4)	0.1†	24
O-1	0.0000	0.2744(8)	0.5000	2.7(1)	6
O-2	0.1666(4)	0.3332(3)	0.5000	3.3(1)	6
O-3	0.2658(2)	0.5316(4)	0.2617(8)	2.1(1)	12
O-4	0.1011(4)	0.4145(4)	0.3184(8)	3.2(1)	24
O-5	0.4258(2)	0.8516	0.2790(10)	2.2(1)	12
O-6	0.1469(4)	0.4795(4)	0.0000	2.5(1)	12
K-2	0.3333	0.6667	0.5000	2*	2*
K-3	0.0000	0.5000	0.5000	2	3.0(1)
K-4	0.0000	0.3222(10)	0.0000	2	5.2(2)
B					
T-1	0.0946(6)	0.3587(8)	0.5000	0.7(1)	12
T-2	0.1658(8)	0.4987(6)	0.2125(10)	1.2(1)	24
O-1	0.0000	0.2724(10)	0.5000	2.2(1)	6
O-2	0.1654(4)	0.3308(10)	0.5000	3.1(1)	6
O-3	0.2650(4)	0.5297(8)	0.2662(14)	1.7(1)	12
O-4	0.1006(5)	0.4148(6)	0.3187(10)	3.5(1)	24
O-5	0.4267(4)	0.8535(8)	0.2770(16)	3.4(1)	12
O-6	0.1464(6)	0.4781(6)	0.0000	2.3(1)	12
K-2	0.3333	0.6667	0.5000	2.0	2*
K-3	0.0000	0.5000	0.5000	2.0	2.9(1)
K-4	0.0000	0.3188(16)	0.0000	2.0	5.3(1)
Pyridine					
D-1	0.1228(18)	0.2129(10)	0.2630	3.4(4)	3.0(2)
C-1	0.0908(18)	0.1967(10)	0.1530	3.4(4)	3.0(2)
N-1	0.1333(18)	0.2192(10)	0.0000	3.4(4)	1.5(1)
C-2	0.0045(18)	0.1534(10)	0.1580	3.4(4)	3.0(2)
C-3	-0.0454(18)	0.1284(10)	0.0000	3.4(4)	1.5(1)
D-2	-0.0212(18)	0.1404(10)	0.2700	3.4(4)	3.0(2)
D-3	-0.1042(18)	0.0992(10)	0.0000	3.4(4)	1.5(1)

Refinement parameters were as follows. A: $R_w = 8.3$, $R_p = 4.48$; $a = 18.6673(1)$, $c = 7.4956(1)$; asymmetry parameter = 0.31(1); $U = 0.58(2)$, $V = -1.03(2)$, $W = 0.69(1)$; no. of variables = 39; range refined = 10–120° 2 θ . B: $R_w = 12.0$, $R_p = 11.3$; $a = 18.6314(7)$, $c = 7.5081(4)$ Å; asymmetry parameter = 0.2(1); $U = 0.62(3)$, $V = -1.00(4)$, $W = 0.68(1)$; no. of parameters = 41; range refined = 10–110° 2 θ . Space group $P6_3/mmm$.

* Constrained.

† Constrained to have same value as T-1.

varied independently, but they showed no evidence of Ga/Si ordering of the kind found in zeolite-A²⁰ for the Si/Al tetrahedral sites. The K⁺ ions in KGaL are located in precisely the sites that they occupy in KAIL²⁸. The final profile (R_p) and weighted profile (R_w) values were 7.5% and 8.3% respectively. Structural details are given in Tables 1A and 2.

Initial refinement of the data for the perdeuteropyridine complex was carried out using the model for KGaL, and a difference Fourier calculation showed that there was appreciable scattering from within the main channels. Recognizing (from infrared evidence³⁰) that the nitrogen of the pyridine is sited close to a K⁺ ion, and that the pyridine probably retains its planarity and other structural features of the free molecule, several model structures were generated—first with the plane of the pyridine initially parallel to (010), then parallel to (001) and to several other angles of tilt with the molecule parallel to (001). A model with the molecule tilted 24° with respect to (010) refined to high occupancy and yielded an encouraging improvement in R_w . Continuing in this manner, and allowing the K-4 site of potassium and framework sites to relax, while retaining N-1, C-3 and D-3 on the (001) mirror plane, a refinement (with an R_p and R_w of 11.3% and 12.00% respectively) was obtained indicating 1.5 molecules of pyridine per unit cell (Fig. 2). At all stages, the pyridine was constrained to the structure of the free molecule³¹. Attempts to refine the structure with the molecule

Table 2 Bond lengths and angles

	KGaL	KGaL + pyridine	
T-1—O-1	1.650(8)	1.691(12)	
T-1—O-2	1.635(15)	1.646(21)	
T-1—O-4(2)	1.695(8)	1.686(13)	
Mean T-1—O	1.669	1.676	
T-2—O-3	1.669(8)	1.687(16)	
T-2—O-4	1.652(8)	1.630(12)	
T-2—O-5	1.664(12)	1.670(12)	
T-2—O-6	1.640(3)	1.639(8)	
Mean T-2—O	1.656	1.656	
T-1—O-1—T-1	132.1	129.0	
T-1—O-2—T-1	152.9	148.2	
T-2—O-3—T-2	135.8	135.3	
T-1—O-4—T-1	141.6	140.9	
T-2—O-5—T-2	139.1	139.7	
T-2—O-6—T-2	153.1	153.7	
Cation coordination to framework oxygens			
K-2—O-3($\times 6$)	2.821(6)	2.821(12)	
K-3—O-5($\times 8$)	2.915(7)	2.897(13)	
K-4—O-4($\times 4$)	2.996(8)	3.014(7)	
K-4—O-6($\times 2$)	2.844(8)	2.855(8)	
Interatomic distances from the pyridine atoms to the zeolite			
N-1—K-4	3.00(3)	D-2—O-1	2.87(2)
C-1—K-4	3.63(3)	C-1—O-1	3.74(3)
C-2—K-4	3.34(2)	C-2—O-1	3.42(2)

No estimated standard deviations were calculated for the framework angles; they are in 1° for each angle.

* In the literature there is talk of another site for the potassium ion, labelled K-1, for which we find no evidence in this study.

placed at other positions, for example as a π complex with K-4, gave a substantially higher R factor ($R_p = 15.5\%$). The final observed and calculated profiles for both KGaL and the zeolite-pyridine complex are shown in Fig. 2. The structural details are given in Tables 1B and 2.

Figure 3A shows the location of a single pyridine molecule in the main channel of zeolite-L. The nitrogen atom is coordinated to potassium, K-4, but the molecule also lies close to the channel wall, thus optimizing its interaction with the zeolite framework. On general chemical principles, coordination of alkali metals by ammine ligands is rare; but clearly the open, 6-fold coordination of K-4 in this dehydrated system offers an attractive location for the basic pyridine molecule. The observed K—N distance, 3.00 (± 0.03) Å, is consistent with metal–nitrogen distances found in amines of NaCl (Na—N ~ 2.6 Å³²).

A comparison of our two refinements shows that the framework structure of the zeolite appears to be essentially unperturbed by the presence of the pyridine molecule, but this conclusion may be misleading because, on average, only 1.5 of the six potassium sites K-4 in each cavity are coordinated. Only a fraction of the framework atoms around the channel will therefore respond to the close proximity of pyridine. However, the position of K-4 does appear to have altered slightly on complexation with the pyridine.

Computer simulation

We have also explored the possibility of calculating the minimum energy position for pyridine in zeolite-L, using atom-atom potentials according to the method of Ramdas *et al.*³³. Kiselev, whose parameterization was used in the present work, has already shown^{34,35} that such calculations yield enthalpies of adsorption that are in good agreement with experimental data. We used the program 'Chemgraf'³⁶. The calculation models the short-range (van der Waals) interactions and the electrostatic terms (dipolar and higher order, in this instance) between the pyridine and the zeolite, but it does not include any covalent contribution to the K—N bond energy—this, of course, is expected to be small. A single molecule was placed as in Fig. 3A and the interaction energy was calculated as a function of its

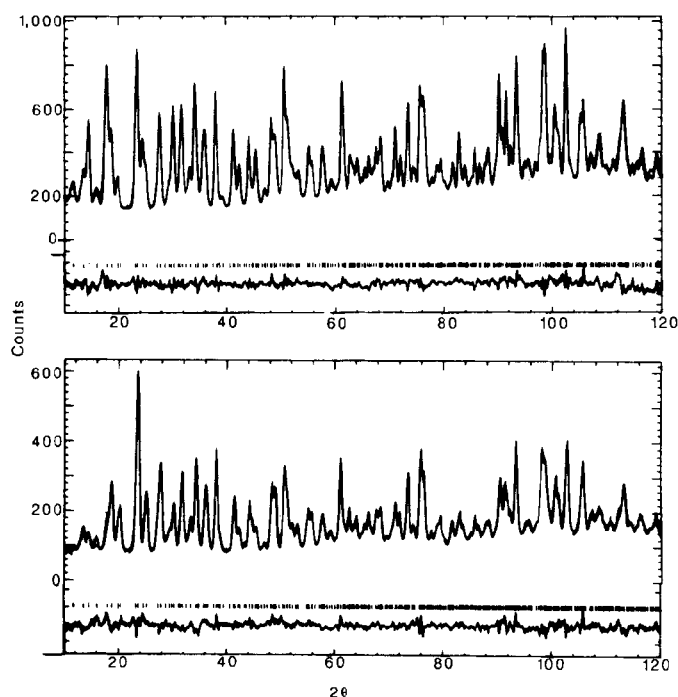


Fig. 2 Neutron powder diffraction profiles of A, KGaL and B, the KGaL-perdeuteropyridine complex. The upper curves show the observed and calculated profiles—these are generally coincident within the resolution of the plotter. The lower curve shows the difference profile. The vertical lines mark the peaks used in refinement (up to 2θ of 120°).

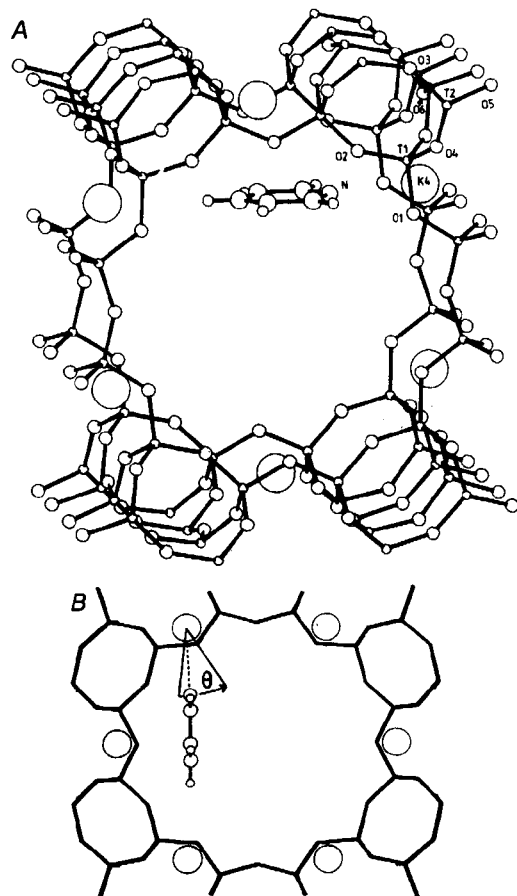


Fig. 3 A, The location of a single pyridine molecule in the large channel of zeolite-L, viewed approximately down $[001]$. B, A view of the pyridine molecule down $[001]$, showing the angle θ which was varied for the calculations shown in Fig. 4A.

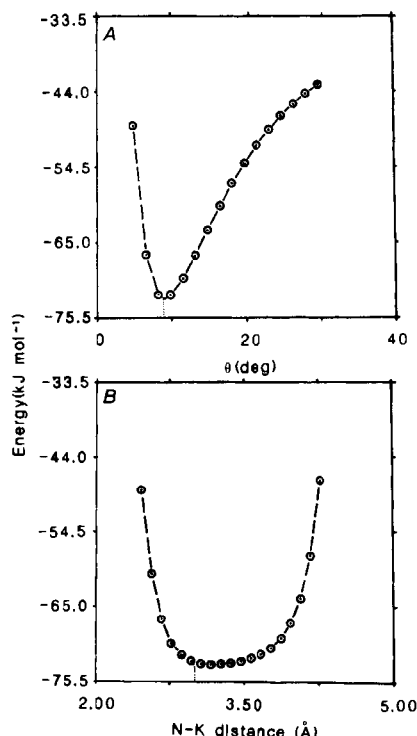


Fig. 4 The interaction energy between pyridine and the zeolite as a function of (A) the angle θ and (B) the K-4—N distance at $\theta = 8.8^\circ$; the observed positions are indicated by dashed lines.

angular position θ (Figure 3B) and the K-4—N distance at $\theta = 8.8^\circ$; the calculations yield an energy minimum at this θ value (Fig. 4A) that is in excellent agreement with the observed value of 8.6° , and a K-4—N distance of $3.13 \text{ \AA}$ (Fig. 4B). The predicted position of the molecule is within $0.2 \text{ \AA}$ of the observed location.

Discussion

It is worthwhile emphasizing that exclusive occupancy of the minimum enthalpy position of the molecule is likely to be found only at very low temperatures, as used in the present work, where entropy considerations are minimized. This factor underlines one of the great advantages of powder neutron diffraction methods—the ease with which data can be collected over a wide temperature range. The concordance between our experimental and theoretical results lends credence to the efficacy of both methods, and more ambitious aims may now be within reach. These include the simulation of catalytic reactions in zeolites, by using a combination of molecular mechanics and molecular orbital calculations, the experimental location of catalytic intermediates, and—again drawing on the enzyme analogy—the study of sorbate motion in zeolites by molecular dynamics.

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New constraints on transient lower mantle rheology and internal mantle buoyancy from glacial rebound data

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A model of mantle rheology which has an explicit transient component of its relaxation spectrum is shown to reconcile two apparently conflicting inferences of lower mantle viscosity. If recent analyses of isostatic geoid anomalies are correct in requiring a large increase of viscosity with depth, then the weak viscosity stratification demanded by postglacial rebound data implies the importance of such rheological behaviour, at least in the Earth's lower mantle.

OVER the past decade a large number of diverse geophysical observations have been shown to be intimately connected with the process of glacial isostatic adjustment. These include relative sea-level histories in the age range 0-18 kyr before present, free-air gravity anomalies over existing centres of postglacial rebound, and certain anomalies in the Earth's rotation including the non-tidal component of acceleration of the axial rotation rate which has recently been confirmed (through J_2) by laser ranging to the LAGEOS satellite^{1,2}, and the wander of the rotation pole with respect to the surface geography which is revealed in the International Latitude Service path to be occurring at a rate near (0.95 ± 0.15) degrees per 10^6 yr towards eastern Canada.

The demonstration of the intimate connection which exists between these phenomena has been provided through systematic application of the linear viscoelastic field theory of glacial isostasy³. Detailed recent accounts of the application of this theory to the four types of data mentioned above have appeared in several recent articles⁴⁻⁷. All of the analyses described in these papers were predicated on the assumption that the mantle could be described as a linear Maxwell solid insofar as its viscoelastic behaviour was concerned. The main result of these analyses has been the demonstration that in the context of this rheological parameterization, the viscosity of the mantle had to be rather uniform, with a lower mantle value of $2-3 \times 10^{21}$ Pa s and an upper mantle value close to 10^{21} Pa s. Furthermore, relative sea-level data from beyond the margin of the Laurentian ice sheet can be invoked⁸ to constrain the thickness of the continental lithosphere to a value near 200 km, and several articles^{5,7,9} have established that in order to explain observed free air gravity anomalies with the same model as is required to fit sea-level observations, the internal density stratification of the mantle must be allowed to deliver a buoyant restoring force when the individual horizons within it are deflected from their equilibrium levels in the isostatic adjustment process. This latter inference is obviously of interest from the point of view of theories of the mantle convection process.

Transient rheology and glacial isostasy

Weertman¹⁰ first drew attention to the possibility of a conflict between the uniform viscosity mantle implied by glacial rebound

data and the highly non-uniform viscosity mantle expected on the basis of the microphysical theory of solid-state creep in the Earth. He suggested that the resolution of this impasse may be simply to allow that the lower mantle viscosity to which the rebound process was sensitive is a transient value rather than the steady-state value which is assumed in the Maxwell analogue. Until recently it has remained equally plausible that the fundamental assumptions underlying the microphysical model might be the cause of the discrepancy between the expected value of the lower mantle viscosity and that required by glacial rebound observations.

Recent analyses of isostatic geoid anomalies by Hager¹¹ and Richards and Hager¹² appear to point to the validity of Weertman's interpretation, however, because these anomalies, presumably forced by the density heterogeneity associated with mantle convection, are interrogating the viscosity spectrum on a characteristic timescale of the order of 10^8 yrs. Richards and Hager found that they could fit the lowest degree components of the geoid height spectrum ($l=2$ and $l=3$) on the basis of the internal heterogeneity of density inferred from seismic tomographic analyses^{13,14} only if the lower mantle were about a factor of 10 more viscous than the upper mantle. Given the simplicity of the model used to make this inference, the increase in the viscosity contrast preferred by the longest wavelength geoid height data above that required by glacial rebound observations may or may not be significant.

In order to obtain a similar reconciliation of geoid height anomalies in the spectral range $4 \leq l \leq 9$, however, Richards and Hager were obliged to add the lateral heterogeneity of density associated with downgoing slabs in oceanic subduction zones to that supplied by objective seismic tomography, since the latter technique is as yet insensitive to these smaller wavelength features when applied on a global basis. To best fit the pattern and amplitude of the slab scale geoid height features then required a very substantial further increase in the contrast of viscosity between upper and lower mantle. Recently stated preferences for the contrast have reached a factor of 10^2 or more. In this article I wish to point out an important implication of this result, if it is in fact correct, and to demonstrate through explicit analysis the way in which it would have to be reconciled with previous work on the problem of glacial

isostatic adjustment if it should be verified by further investigation.

There have in fact been several recent re-analyses of post-glacial rebound phenomena, inspired in part by this result, using a rheological parameterization which explicitly includes transient relaxation of the lower mantle as was suggested originally by Weertman¹⁰ to be necessary on other grounds. If such additional rheological complexity were manifest it would clearly explain in a rather simple way why the viscosity of the lower mantle 'seen' by short timescale glacial rebound phenomena might be significantly lower than the viscosity controlling the longer timescale mantle convection process. The Burgher's body model of Peltier *et al.*¹⁵ has recently been used by several authors¹⁶⁻¹⁸ to represent the hypothesized lower mantle transient. The main conclusion derived from this work is that such models might provide equally acceptable fits to the data as those previously delivered by the Maxwell models in which the relaxation spectrum contains no transient component. In this article I demonstrate that, when all of the signatures of the isostatic adjustment process are invoked simultaneously, it is possible to restrict very considerably the class of such transient models allowed by the data. In particular it will be demonstrated that acceptable models in the allowed class are those which essentially behave as Maxwell models and that strong internal buoyancy is still required within the mantle if they are to reconcile simultaneously both relative sea-level and free-air gravity observations.

Burgher's body rheology

The frequency-dependent shear modulus of a three-dimensional Burgher's body rheology has the form¹⁵:

$$\mu(s) = \frac{\mu_1 s}{(s + \mu_1/\nu_1)} \left[\frac{(s + \mu_2/\nu_2)(s + \mu_1/\nu_1)}{(s + \mu_2/\nu_2)(s + \mu_1/\nu_1) + \mu_1 s/\nu_2} \right] \quad (1)$$

in which s is the Laplace transform variable, μ_1 is the elastic shear modulus, ν_1 the steady-state viscosity, and μ_2 and ν_2 are the shear modulus and viscosity of the Kelvin-Voigt element of the Burgher's body which determine the strength and timescale of the transient relaxation which it supports. Note from equation (1) that in the limit $\nu_2 \rightarrow \infty$ the Burgher's body degenerates to a Maxwell solid with $\mu(s) = \mu_1 s/(s + \mu_1/\nu_1)$ which is the same analogue as has been conventionally used in postglacial rebound analyses³. More important for our present purposes is the limit $\mu_2 \rightarrow 0$ in which case the analogue again degenerates to a Maxwell solid but in this limit $\mu(s) = \mu_1 s/(s + \mu_1/\nu_{\text{eff}})$ where the effective viscosity $\nu_{\text{eff}} = \nu_1 \nu_2/(\nu_1 + \nu_2)$. This is important because it means that in the limit of large defect ($\mu_2 \ll \mu_1$) the relaxation will proceed as if it were a steady-state relaxation, and to the extent that $\nu_1 \gg \nu_2$ the effective viscosity governing the rate at which relaxation proceeds will be the transient viscosity since $\nu_{\text{eff}} \approx \nu_2$ in this limit. This article addresses the question of whether transient rheologies which have only modest elastic defect, for which the condition $\mu_2 \ll \mu_1$ does not apply, are viable models for the explanation of isostatic adjustment data. The results presented below demonstrate that they are not and therefore that previous inferences of effective lower mantle viscosity based upon application of the Maxwell analogue may simply be interpreted as representing a transient element of the viscosity spectrum if this should prove necessary. Although transient models which are allowed by the data are able to fit free-air gravity observations with somewhat less internal mantle buoyancy than was required with the Maxwell analogue, non-negligible buoyancy is still required.

For purposes of the present discussion we will assume that only the lower mantle has a transient component in its relaxation spectrum and therefore will take $\nu_2 = \infty$ in the upper mantle over the depth range 0–670 km. This is not meant to imply that transient relaxation of the upper mantle can be excluded on any *a priori* grounds but only that we intend to illustrate the influence of transient rheology in terms of a model in which its effect is confined to the lower mantle. We will also assume that the steady-state viscosity of the upper mantle is 10^{21} Pa s and

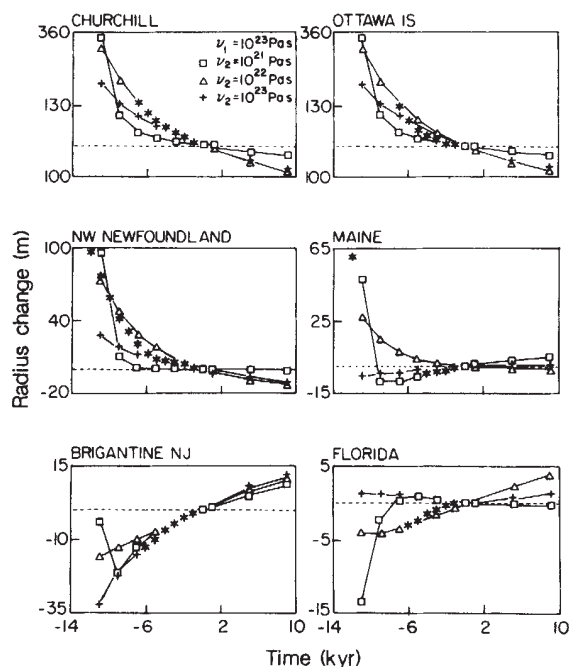


Fig. 1 Comparison of observed (*) and predicted relative sea-level data at six North American sites for the Burgher's body rheology with $\mu_2/\mu_1 = 0.1$. The lithospheric thickness of the Burgher's body is fixed at 120.7 km, $\nu_1 = 10^{21}$ Pa s and $\nu_2 = \infty$ in the upper mantle, and $\nu_1 = 10^{23}$ Pa s in the lower mantle. The different relative sea-level predictions on each plate are for models with lower-mantle ν_2 values of: 10^{21} Pa s ($\square$), 10^{22} Pa s ($\triangle$), 10^{23} Pa s (+).

the lithospheric thickness 120.7 km. Between the seismic discontinuity at 670 km depth and the core-mantle boundary, we will, for purposes of illustration, accept the results of Richards and Hager¹² as fixing the steady-state creep resistance of the lower mantle to the value of 10^{23} Pa s, that is, two orders of magnitude higher than the upper mantle value. We will then invoke the four previously discussed signatures of the isostatic adjustment process to constrain the remaining parameters μ_2 and ν_2 which are required to complete the description of the rheology of the lower mantle. The elastic structure upon which the above viscous layering is superimposed will be assumed identical to the model with two internal mantle density discontinuities which I have used previously⁸ which as a 3.8% increase of density at 420 km depth and a 6.2% increase at 670 km depth. In the analysis of free-air gravity anomalies we will compare the predictions of this model to one which has no internal mantle density discontinuities and therefore no source of internal mantle buoyancy.

Transient relaxation and postglacial rebound

Figure 1 shows observed and predicted relative sea-level variations at six North American sites for the Burgher's body rheology with $\mu_2/\mu_1 = 0.1$ and for three different choices of the lower mantle ν_2 ($= 10^{21}$ Pa s, 10^{22} Pa s and 10^{23} Pa s) with other parameters of the model fixed to the values stated above. All calculations are based upon the circular disk load approximation to the Laurentian ice sheet with parameters listed in ref. 8 and include the influence of seven prior 10^5 -yr cycles of glaciation and deglaciation as may be inferred to have occurred on the basis of $\delta^{18}\text{O}$ data from deep-sea sedimentary cores. Inspection of these comparisons demonstrates that at sites within the ice sheet margin like Churchill, Ottawa Islands, and north-west Newfoundland, the data prefer 10^{21} Pa s $< \nu_2 < 10^{22}$ Pa s with the lowest value strongly rejected because it leads to overly rapid initial relaxation and thus severely underestimates present day emergence rates. More detailed analysis demonstrates that the preferred ν_2 at Churchill is 5×10^{21} Pa s, whereas that at both the Ottawa Islands and north-west Newfoundland sites is closer to $2-3 \times 10^{21}$ Pa s. At the Maine site which is on the ancient

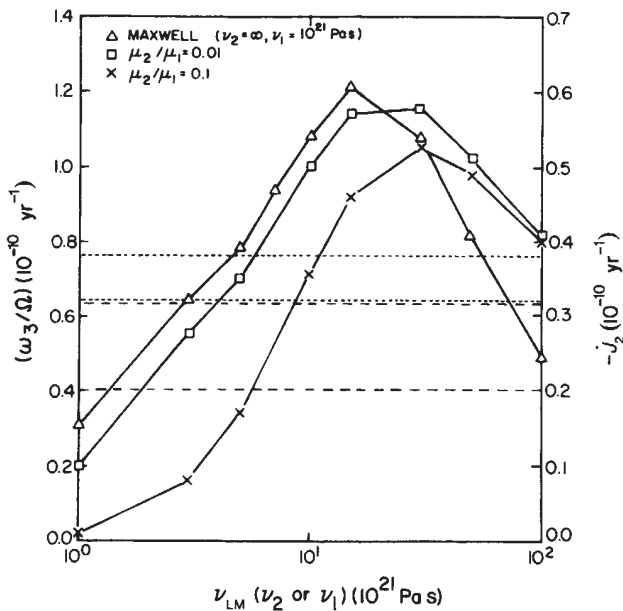


Fig. 2 Predicted J_2 and the associated non-tidal acceleration of rotation as a function of lower-mantle viscosity for both the Burgher's body rheologies with $\mu_2/\mu_1 = (0.1, 0.01)$ and for the Maxwell analogue to which the Burgher's body degenerates in the limit $\mu_2 \rightarrow 0$. The upper and lower horizontal dashed lines on this plate correspond respectively to the observed values based upon the analyses of Lageos data by Yoder *et al.*¹ and Rubincam².

margin of the Laurentian ice sheet, high values of ν_2 eliminate the non-monotonic signature from the predictions and therefore are excluded by the data. As was found to be the case with the Maxwell analogue, even a value of 5×10^{21} Pa s for lower mantle ν_2 is unacceptable from the point of view of such data. At Brigantine further south (see ref. 8 for a location map) the data are not as sensitive to lower-mantle viscosity. As shown previously⁸, however, the observations at such sites which are near the crest of the glacial forebulge are extremely sensitive to lithospheric thickness. The fact that we are able to fit the observations at this site with a model that has a lithospheric thickness of 120.7 km suggests that thinner lithospheres may be allowed in models which include lower-mantle transient relaxation.

Further south at Florida the observations do require some elevation of lower-mantle viscosity in order to eliminate the prediction of raised beaches which would otherwise obtain. This is also in accord with results obtained with the Maxwell model¹⁸ and detailed analysis for a wide range of ν_2 values demonstrates that a value of $\nu_2 \approx 3 \times 10^{21}$ Pa s is sufficient for this purpose as well as being allowed by the observations at edge sites like Maine. The main result implied by these analyses of relative sea-level data is the demonstration that the transient Burgher's body model can reconcile glacial isostatic adjustment data to a certain extent, and if $\mu_2/\mu_1 = 0.1$ the model is already behaving very similarly to the Maxwell model, since the lower-mantle value of ν_2 inferred by fitting the model to the data has the same value ($\sim 3 \times 10^{21}$ Pa s) as was previously inferred for ν_1 in the context of the Maxwell analogue. It is rather more difficult to fit all relative sea-level data with the same Burgher's body model than has previously been shown to be the case with the Maxwell model, however, and, as we will proceed to demonstrate, even the small value of $\mu_2/\mu_1 = 0.1$ is too large to accommodate the remaining signatures of the rebound process.

Figure 2 shows observed and predicted J_2 for the three-disk load approximation to the deglaciation history which is detailed in ref. 6, and which contains about 100 m of mean sea-level variation between successive glacial maxima and minima. Again the calculations are based upon the assumption that seven previous 10^5 -yr cycles of glaciation and deglaciation have occurred. The upper horizontal dashed lines on Fig. 2 denote the bounds on J_2 recently inferred by Yoder *et al.*¹, whereas the

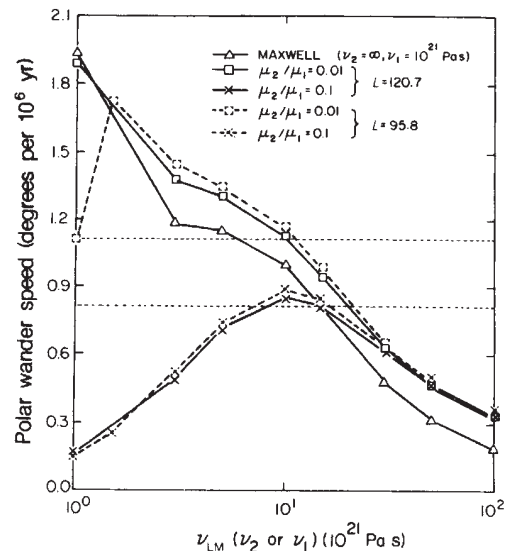


Fig. 3 Predicted polar wander speed as a function of lower-mantle viscosity for the Burgher's body rheologies with $\mu_2/\mu_1 = (0.1, 0.01)$ in which case the lower-mantle viscosity varied as the transient value ν_2 , and for the Maxwell model to which the Burgher's body degenerates in the limit $\mu_2 \rightarrow 0$ in which case the lower-mantle viscosity varied is the steady-state value ν_1 . The solid curves on this figure are predictions for models with lithospheric thickness 120.7 km while the dashed curves are the predictions of models with lithospheric thickness of 95.8 km.

lower lines denote the bounds on the same observations by Rubincam². Predictions are shown as a function of lower-mantle viscosity for three different models. The first model is the Maxwell model which I have used in previous analyses of this datum and the lower-mantle viscosity varied in these calculations is therefore the steady-state viscosity ν_1 . The second and third models are Burgher's body analogues with $\mu_2/\mu_1 = 0.1$ and 0.01 and the lower-mantle viscosity varied in these calculations is the transient viscosity ν_2 since the steady-state value ν_1 is fixed to the value of 10^{23} Pa s on the basis of the results of Richards and Hager¹². As pointed out in ref. 19, the J_2 observation is an especially important one because it is relatively insensitive both to the amount of internal buoyancy in the mantle and to the thickness of the lithosphere. It should therefore deliver a particularly unambiguous constraint upon the contrast in viscosity between the upper and lower mantle. Insofar as the J_2 observation is concerned, the ratio $\mu_2/\mu_1 = 0.1$ is still too large for the Burgher's body to have degenerated to an equivalent Maxwell solid. For $\mu_2/\mu_1 = 0.01$, however, the predictions of the Burgher's body as a function of lower-mantle ν_2 are very close to the predictions of the Maxwell solid as a function of lower mantle ν_1 . Most important, however, in the fact that with $\mu_2/\mu_1 = 0.1$ the lower-mantle value of ν_2 must be near 10^{22} Pa s in order to fit the observed J_2 . This is not compatible with the requirements of the sea-level data discussed previously (particularly those at edge sites like Maine), so that in the context of the Burgher's body $\mu_2/\mu_1 < 0.1$ seems to be required. Only if the transient model satisfies the asymptotic condition and thus acts effectively as a Maxwell model is such a model acceptable. This is further confirmed by the second rotational datum.

Figure 3 shows an analogous comparison to that in Fig. 2 but for the speed of polar wander predicted by the three ice cap model⁶. As discussed elsewhere^{6,7}, this is a particularly intricate calculation to perform as the forced polar wander involves a complex interplay between rotational and isostatic adjustment effects. The horizontal dashed lines on Fig. 3 represent the observed speed of polar wander of 0.95 ± 0.15 deg per 10^6 yr. The predicted polar wander direction does not depend at all on the rheology⁶. Also shown for comparison purposes, are results for the two Burgher's body rheologies with a reduced lithospheric thickness of 95.6 km which are shown as the dashed line.

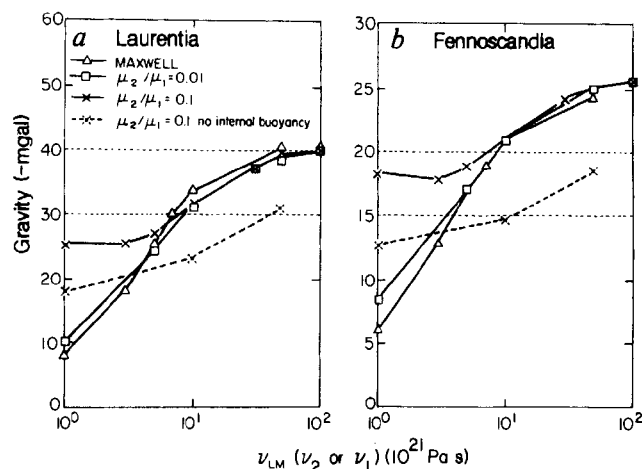


Fig. 4 Predicted peak free-air gravity anomalies at the centres of Laurentia (a) and Fennoscandia (b) as a function of lower-mantle viscosity in the Burgher's bodies with $\mu_2/\mu_1 = (0.1, 0.01)$ in which cases the lower-mantle viscosity varied as the transient value ν_2 , and in the Maxwell analogue to which the Burgher's body converges in the limit $\mu_2 \rightarrow 0$, in which case the lower-mantle viscosity varied as the steady-state value ν_1 . The elastic basic state used in these calculations is the one in which there are density increases of 3.8% and 6.2% at 420 km and 670 km depth respectively. Also shown on these separate plates by the dashed lines are the predicted free-air gravity anomalies for the Burgher's body analogue with $\mu_2/\mu_1 = 0.1$ using an elastic basic state in which the mantle contains no internal buoyancy. Clearly, even in the Burgher's body case considerable internal buoyancy appears to be required to fit the observed free-air anomalies but perhaps somewhat less than for the Maxwell model.

Again, the results for $\mu_2/\mu_1 = 0.01$ are close to the predictions of the Maxwell model to which the Burgher's body degenerates in the limit $\mu_2 \rightarrow 0$. For the transient model with $\mu_2/\mu_1 = 0.1$, however, the polar wander speed is sharply reduced, so much so that it cannot match the observation whatever the value of lower-mantle ν_2 . Indeed, only for the high transient model with $\nu_2 = 10^{22}$ Pa s is it barely within a standard error of the observed speed of 0.95 deg. per 10^6 yr. This datum also requires large viscosity contrast with $\mu_2/\mu_1 = 0.1$, if it can fit the data at all, and therefore is ruled out by the sea-level data, particularly those at edge sites which display a non-monotonic history of postglacial sea-level variation. The predictions of this signature of the response for the model with thinner lithosphere and $\mu_2/\mu_1 = 0.01$ reveals the same sensitivity as previously demonstrated²⁰ for the Maxwell model. For the larger value of $\mu_2/\mu_1 = 0.1$, however, this sensitivity is absent. It is clearly because of this sensitivity to lithospheric thickness variations that the Maxwell and $\mu_2/\mu_1 = 0.01$ models can be made to fit the observed polar wander speed datum with the small viscosity contrast required by sea-level observations.

Figure 4 shows peak present-day predicted free-air gravity anomalies at the centre of the Laurentian and Fennoscandian disk loads as a function of lower-mantle viscosity for the Maxwell model and for the Burgher's bodies with $\mu_2/\mu_1 = 0.1$ and 0.01 which degenerate to it in the limit $\mu_2 \rightarrow 0$. Inspection of these comparisons again shows that the predictions of the Burgher's body with $\mu_2/\mu_1 = 0.01$ are almost identical to those of the Maxwell model for all values of lower-mantle viscosity. For $\mu_2/\mu_1 = 0.1$, however, the present-day free-air gravity

anomalies predicted by the Burgher's body model are considerably enhanced at low values of ν_2 , implying that one effect of transient lower-mantle rheology may be to allow one to fit the observed anomalies with much lower viscosity contrasts than would otherwise be required. In fact if $\mu_2/\mu_1 = 0.1$ were acceptable on other grounds, which we have shown to be unlikely, one could fit the observed anomaly in Fennoscandia without any viscosity contrast at all and the contrast required in Laurentia would be somewhat reduced. The dashed lines on Fig. 4 show the size of the free-air gravity anomaly which would be expected in these two regions if the mantle contained no source of internal buoyancy within its volume. These predictions are shown only for the Burgher's body with $\mu_2/\mu_1 = 0.1$ and demonstrate that even if this value of μ_2/μ_1 were acceptable, the influence of transient rheology would not completely remove the requirement established previously for the Maxwell model that the density discontinuities at 420 km and/or 670 km depth in the mantle be capable of inducing a buoyancy force when they are deflected from their equilibrium positions by the ice sheet loading process. Peltier²¹ and Peltier *et al.*¹⁸ have provided detailed discussions of the importance of this observation from the point of view of several geodynamic processes including that of mantle convection.

Transient rheology and internal buoyancy

The new calculations described above have reinforced the results of recent analyses based on the suggestion of Weertman¹⁰ that the lower-mantle viscosity to which postglacial rebound is sensitive may be a transient rather than a steady-state value. They do also establish, however, that only transient rheologies in which the elastic defect is very large are candidate rheologies which are allowed by the isostatic adjustment data. Since such rheologies operate essentially as Maxwell rheologies with an effective viscosity $\nu_{eff} = \nu_1 \nu_2 / (\nu_1 + \nu_2)$, it appears that there is very little to be gained in using the more complicated Burgher's body rheology of Peltier *et al.*¹⁵ to describe isostatic adjustment processes. One simply thereby adds additional parameters to the model which are not required by the data. One extremely important implication of the results obtained here is that introduction of transient relaxation into the lower mantle cannot, apparently, completely relieve the requirement that the internal mantle density stratification be able to deliver some buoyant restoring force when it is deformed in the isostatic adjustment process. Without such internal buoyancy in the mantle it is apparently not possible to reconcile observed free-air gravity anomalies with the same viscoelastic model as is required by relative sea-level observations. As discussed in ref. 21, this point is important since it bears on the question of the chemical stratification of the mantle and/or on the extent to which mantle phase transitions behave in a univariant fashion.

In order to make further progress in constraining the extent to which transient relaxation may be required to explain isostatic adjustment observations, we clearly need an independent re-analysis of the conclusions of Richards and Hager¹² concerning the implications of observed isostatic geoid anomalies. If it could be shown that their slab scale anomalies did not in fact require large viscosity stratification, this would demonstrate that no significant transient component of the mantle relaxation spectrum in fact exists. By combining in this way both long-timescale and short-timescale phenomenology which are sensitive to the relaxation process, we shall eventually be able to refine considerably our understanding of the physical mechanisms by which relaxation actually proceeds.

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Structure of the protein subunits in the photosynthetic reaction centre of *Rhodopseudomonas viridis* at 3 Å resolution

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The molecular structure of the photosynthetic reaction centre from Rhodopseudomonas viridis has been elucidated using X-ray crystallographic analysis. The central part of the complex consists of two subunits, L and M, each of which forms five membrane-spanning helices. We present the first description of the high-resolution structure of an integral membrane protein.

The first steps in photosynthesis are mediated by protein-pigment complexes in the photosynthetic membrane. Light energy is absorbed primarily by light-collecting complexes and transferred to reaction centres where it is used with a very high quantum efficiency to transport electrons across the membrane. The reaction centre from the purple bacterium *Rhodopseudomonas viridis* consists of the four protein subunits L, M, H and c-type cytochrome¹ with four covalently linked haem groups². Other prosthetic groups associated with the reaction centre are four bacteriochlorophyll *b* (BChl-*b*), two bacteriopheophytin *b* (BPh-*b*), two quinones, one non-haem ferrous iron and carotenoid(s)¹. Apart from the cytochrome, which has been found as an integral constituent of reaction centre complexes only in some bacteria, it is generally accepted that reaction centres of purple bacteria are organized in a very similar way. This can be concluded from the subunit composition, from homology between the amino-acid sequences of the L and M subunits, from chromophore composition and from similar spectroscopic and electron-transfer properties (for reviews see, for example, refs 3–7).

The reaction centre from *Rps. viridis* was the first to be crystallized⁸; the photochemical activity of the crystalline material has been demonstrated⁹. An X-ray structure analysis of these well-ordered crystals allowed the calculation of an electron-density map at 3 Å resolution; an atomic model of the prosthetic groups was deduced from this map¹⁰. The arrangement of the pyrrole ring systems of BChl-*b* and BPh-*b* molecules in the central region of the reaction centre (assumed to consist of the subunits L and M) shows an approximate 2-fold symmetry with two closely associated BChl-*b*s near the symmetry axis. These two molecules form the 'special pair' postulated from the results of spectroscopic investigations¹¹. The special pair is symmetrically in contact with two BChl-*b* molecules, each of which has a BPh-*b* as a neighbour. These chromophores form two branches that are apparently suitable as electron pathways. The right-hand side branch (see Fig. 4 in ref. 10) ends with a quinone close to the non-haem iron located on the local symmetry axis. The second quinone and the carotenoid are either not present or disordered in the crystal. The four haem of the cytochrome show a roughly linear arrangement with one haem near the special pair¹⁰.

This chromophore model correlates well with the electron-transfer steps determined from spectroscopy¹². After absorption of light an electron is transferred from the excited primary electron donor (the special pair), possibly via a BChl-*b*¹³ to an intermediate acceptor (BPh-*b*), and further to a primary quinone acceptor (Q_A or tightly bound quinone). Q_A reduces a secondary quinone acceptor (Q_B or loosely bound quinone). Q_B , when fully reduced and protonated, is commonly assumed to leave the reaction centre, and to be replaced from a quinone pool in the membrane¹⁴. The oxidized special pair is reduced by the cytochrome.

Here we report further interpretation of the electron-density map. We describe the folding of the polypeptide chains of the protein subunits in the reaction centre and the spatial relation between protein and prosthetic groups. With this structure we can present the first model of an integral membrane protein determined at nearly atomic resolution.

Electron-density map

The reaction centre crystals belong to the space group $P4_32_12$ with unit cell constants of $a = b = 223.5$ Å, $c = 113.6$ Å, and contain one centre per asymmetrical unit^{8,10}. The electron-density map at 3 Å resolution was calculated with phases from multiple isomorphous replacement using five heavy-atom derivatives; this map was improved further by solvent flattening¹⁵. A detailed report on data collection, map calculation and arrangement of prosthetic groups is described elsewhere¹⁰.

The chromophore model facilitated the identification of the protein subunits of the reaction centre. The haem groups are evidently bound to the cytochrome subunit; BChl-*b*, BPh-*b*, non-haem iron and quinone are known to be associated with the subunits L and M in reaction centres from purple bacteria^{4,16,17}. Available amino-acid sequences of 46, 30 and 28 residues¹⁸ at the amino-termini of the subunits L, M and cytochrome, respectively, could be located in the map. The H subunit was identified on the basis of a partial gene sequence corresponding to 68 residues from the amino terminus.

For model building of the protein subunits a minimap (scale 2 mm Å⁻¹), and interactive display systems (Vector General 3400 and Evans & Sutherland PS330) driven by the program FRODO¹⁹ were used. Optimum fit of helices and large side chains to the density was achieved with the real-space refinement option in FRODO²⁰. Segments with unknown amino-acid sequence were built as polyalanine. Residue numbers were prefixed with the letters L, M, C and H, respectively, for distinction between subunits.

The good quality of the map allowed unambiguous chain tracing even in the absence of amino-acid sequence information. Recently the complete amino-acid sequences of the subunits H²¹, L and M (H.M., K. A. Weyer, H. Gruenberg, I. Dunger, D. Oesterhelt and F. Lottspeich, in preparation) became available from the corresponding gene sequences and were incorporated into the model. The amino-acid sequence of the cytochrome, presently being investigated with protein-chemical methods (K. A. Weyer, H.M. and F. Lottspeich, unpublished data), is still incomplete. However, peptide sequences resulting from this work could be located in the electron density and used for model building. Currently, these pieces add up to ~60% of the residues. The absence of a contiguous sequence for the cytochrome leads to an uncertainty in the residue numbering for this subunit caused by possible erroneous omission or insertion of residues during chain tracing.

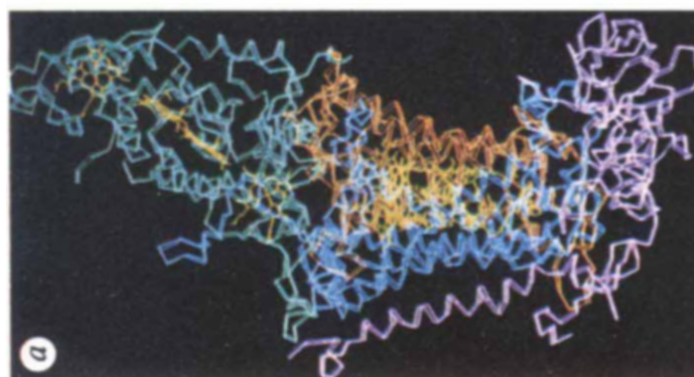
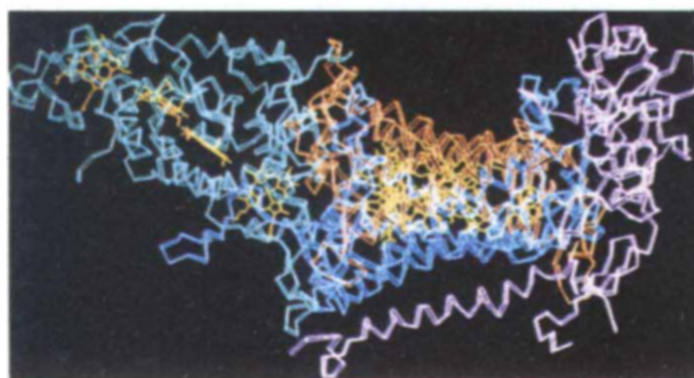
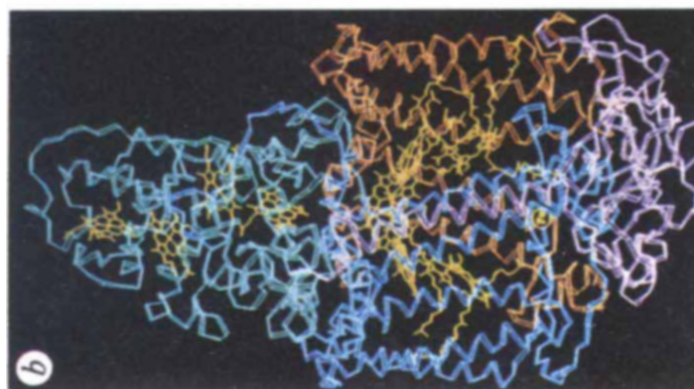
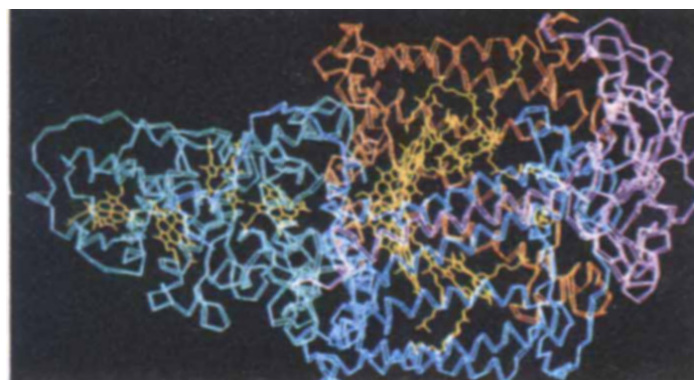
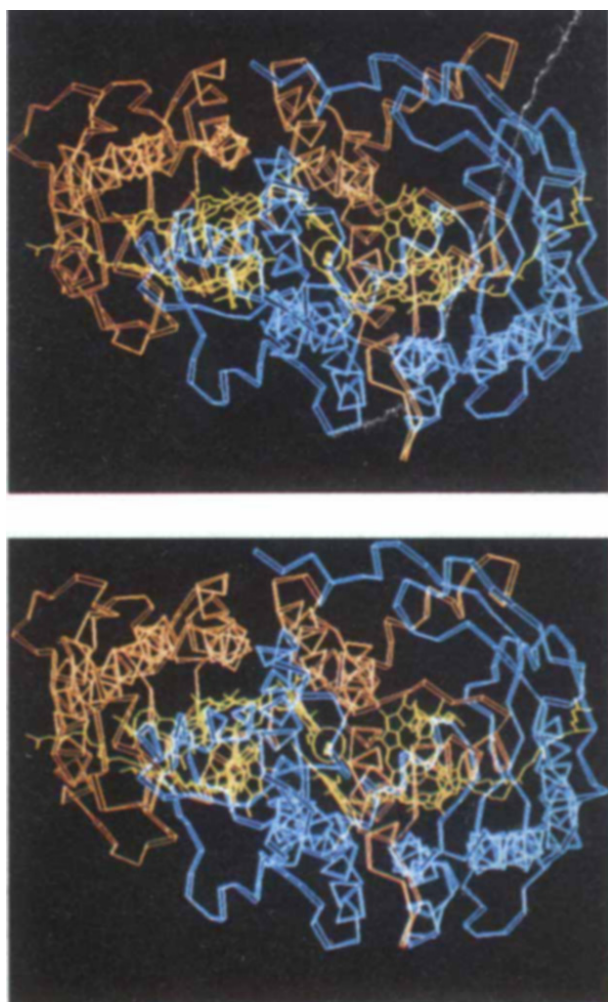


Fig. 1 (Parts *a*, *b* above.) Stereo drawing of the complete reaction centre complex in two orientations. Panel *a* is rotated with respect to *b* by 90° around a vertical axis. Green, cytochrome; orange, L; blue, M; purple, H; yellow, prosthetic groups. Figs 1-5 were produced by a computer program written by A. M. Lesk and K. D. Hardman⁵⁰.

Fig. 3 (Left) Stereo drawing of the L-M complex and associated chromophores viewed along the L-M local diad. Protein chains are represented as ribbons. Colours as in Fig. 1.

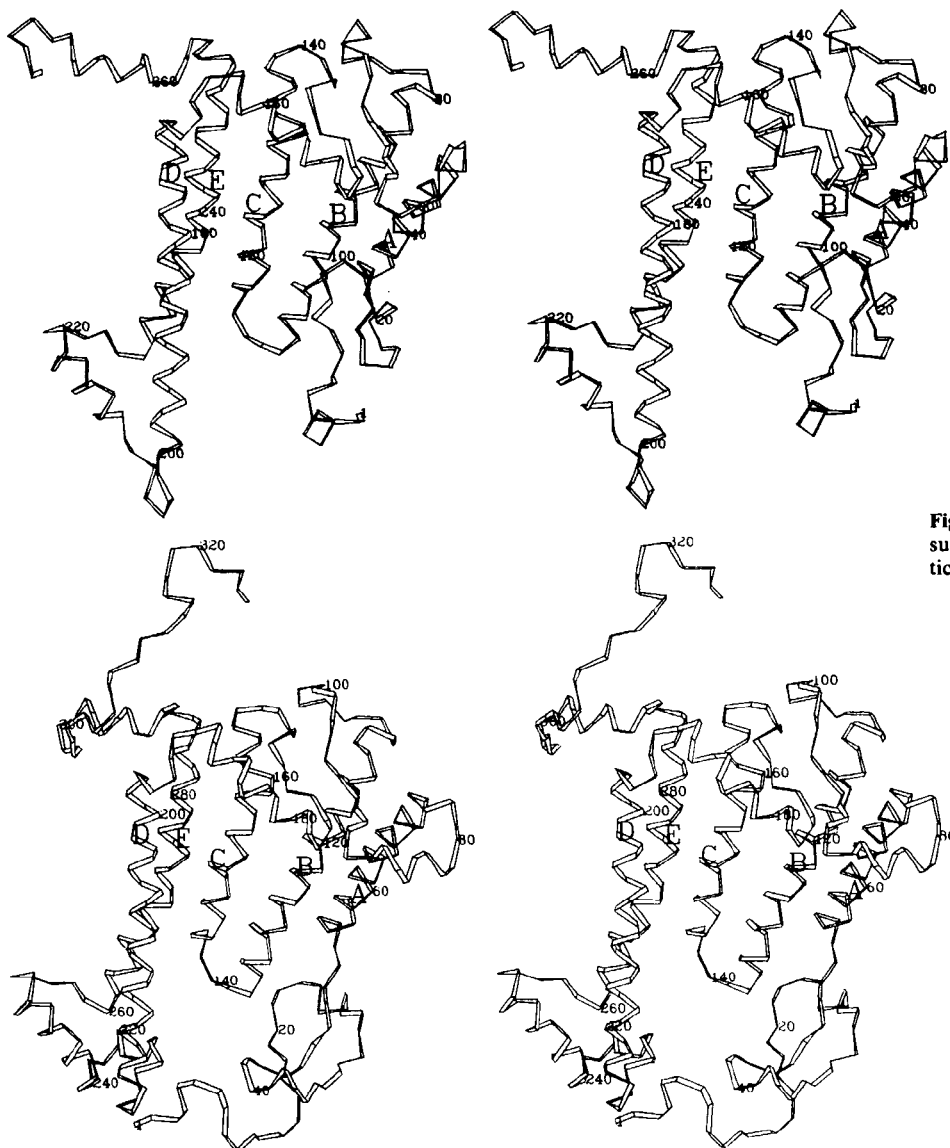


Fig. 2 Stereo drawings of *a*, subunit L; *b*, subunit M represented as ribbons in identical orientations. Transmembrane helices A-E are labelled.

A crystallographic R -value ($R = \sum ||F_{\text{obs}}| - |F_{\text{calc}}|| / \sum |F_{\text{obs}}|$) was calculated when the model contained ~70% of the correct sequence. For 46,658 reflections between 7- and 3-Å resolution we obtained $R = 0.359$; this value is exceptionally low for a protein model before crystallographic refinement.

The reaction centre complex

The course of the polypeptide chains and the location of the prosthetic groups of the centre are shown in Fig. 1. The central part consists of the subunits L and M which bind the BChl-*b*, BPh-*b*, the quinone and the non-haem iron. The cytochrome subunit is bound to a flat surface of L-M close to the special pair of BChl-*b*. A large contact area and the carboxy-terminal arm of the M subunit are responsible for the tight binding of the cytochrome to L-M. The major part of the H subunit is in contact with the other flat surface of the L-M complex that is close to the binding sites of the quinone and the non-haem iron. The amino-terminal segment of H forms a helix extending from the cytochrome side along the surface of the M subunit. The longest dimension of the reaction centre complex is ~130 Å between the tips of cytochrome and H subunit.

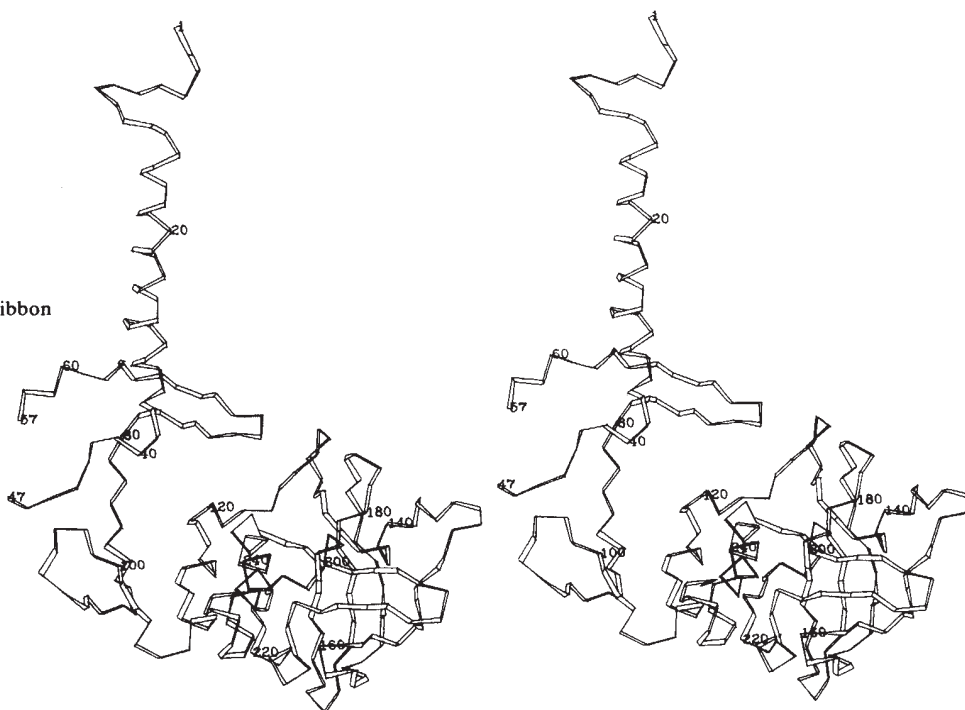
In our electron-density map there is no clear indication of the presence of ordered lipid or detergent that would provide direct evidence on the position and orientation of the membrane relative to the reaction centre complex. However, the highly hydrophobic surface of the central region of the centre indicates that this part (that is, the subunits L and M and the amino-

terminal helix of H) spans the membrane. The same conclusion follows from the fact that with electron transfer from the special pair to the quinone(s) a charge separation across the membrane takes place. Recently, the amino-terminus of the L-subunit was localized at the cytoplasmic side of the photosynthetic membrane from *Rhodospirillum rubrum*²². Assuming the same orientation for the reaction centre from *Rps. viridis*, this means that the cytochrome is located at the periplasmic side, and the major part of the H subunit is at the cytoplasmic side of the membrane.

Subunits L and M

Subunit structure. Although L and M differ in length by 50 residues (L, 273 residues; M, 323 residues), their folding is generally very similar. Using the method of Rossmann and Argos²³, 219 C α -positions of M could be rotated onto corresponding positions of L to an r.m.s. distance of 1.25 Å. The rotation axis is defined by the polar angles $\phi = 43^\circ$, $\psi = 39.4^\circ$, $\kappa = -179.3^\circ$ (where ϕ is the angle between the rotation axis and y -axis; ψ is the angle between projection of the rotation axis on x - z plane and x -axis; and κ is the rotation angle). At the present level of accuracy this L-M local diad is virtually identical to the local diad relating ring atoms of BChl-*b* and BPh-*b* ($\phi = 43^\circ$, $\psi = 37.6^\circ$, $\kappa = -177.1^\circ$)¹⁰. Figure 2 shows ribbon drawings of subunits L and M in the same orientation. Outstanding structural features of both subunits are five long helical regions (mostly α -helix) numbered A-E (LA-LE in the L subunit.

Fig. 4 Stereo drawing of subunit H (ribbon representation).



MA-ME in the M subunit). They consist mainly of hydrophobic residues, many of which are exposed to the environment. Therefore, we assume that these helices span the membrane; their lengths range between 24 and 30 residues (A, L32-L55, M52-M78; B, L84-L112, M110-M139; C, L115-L140, M142-M167; D, L170-L199, M197-M225; E, L225-L251, M259-M285). The helices are arranged side by side in a concave layer in the order A, B, C, E and D, so that helices C and E are neighbours and run approximately parallel, whereas all other neighbouring helix pairs within a subunit run roughly antiparallel. The angles between the helix axes and the L-M local diad are $<25^\circ$ for the helices A, B, C and E and $\sim 38^\circ$ for helix D.

On both sides of the helical region the remaining segments of L and M form two flat surfaces in intimate contact with the subunits cytochrome and H, respectively. The surface in contact with the H subunit consists of the amino-terminal segments and the helix connections D-E; helix connections B-C are short turns which also come close to this surface. Helix connections A-B and C-D and carboxy-terminal chain segments contribute to the cytochrome contact surface. Apart from the transmembrane helices, further segments with helical conformation are located in both contact surfaces: L208-L220, M36-M42, M240-M255 at the H contact surface and L149-L164, L259-L269, M80-M88, M178-M192 and M281-M300 at the cytochrome contact surface. Only at the amino termini of L and M two-stranded antiparallel β -sheets are found comprising segments L24-L26, L29-L31 and M27-M35, M44-M51, respectively.

Major structural differences between L and M are observed at or near the contact surfaces. From the amino terminus to the start of transmembrane helix A, M is 20 residues longer than L; these additional residues mainly contribute to the two-stranded antiparallel β -sheet at the H contact surface. The helix connection D-E of M, which is also at the H contact surface, is seven residues longer than in L. At the cytochrome contact surface the helix connection A-B is seven residues longer in M than in L. From the end of helix E to the carboxy terminus M is 16 residues longer than L; the additional residues of M form the arm that folds along the surface of the cytochrome, and appears to contribute to the tight binding of this subunit in the reaction centre complex.

L-M association. The subunits L and M cohere predominantly at or near the H contact surface. Helices D, E and the C-D connection of one subunit intercalate between helices C and E

in the other subunit (see Fig. 3). Helices LD, LE, MD and ME come close to the L-M local diad, and are in contact with each other (see Fig. 1). The non-haem iron is bound between these four helices, each of which contributes one ligand to the iron. Further L-M contacts at the H contact surface are contributed by the helix connections D-E and by the amino-terminal segments (Fig. 3).

Near the cytochrome contact surface a gap opens up between helix pairs LD, LE and MD, ME to accommodate the special pair on the L-M local diad. Helices C and E are bent away from the L-M local diad near the cytochrome contact surface, and so increase the space available for the accessory BChl-*b* and the BPh-*b* molecules. Both L and M contribute one half of the cytochrome contact surface with apparently weak lateral contacts between these halves. At the intersection of the L-M local diad and the cytochrome contact surface the side chain of residue Tyr L162 is located between the closest haem group and the special pair; it may be involved in electron transfer.

With its two only partially overlapping layers of transmembrane helices the L-M complex has a remarkably non-globular structure in which the surface area accessible from the environment appears to be significantly larger than in globular water soluble proteins. Its shape suggests that the contact surfaces are parallel and that the L-M local diad is perpendicular to the membrane plane, in agreement with chromophore orientations determined optically in oriented *Rps. viridis* cells^{24,25}.

Binding of prosthetic groups in L-M. As reported previously¹⁰, protein side chains are bound to the Mg^{2+} ions of BChl-*b*; all four BChl-*b* ligands are histidines as most BChl-*a* ligands found in the BChl-*a* protein from *Prosthecochloris aestuarii*^{26,27}. The BChl-*b*s are designated BC_{LP} , BC_{MP} , BC_{LA} and BC_{MA} , where L and M refer to the ligation and P and A indicate 'special pair' and 'accessory', respectively. The Mg^{2+} ligands of BC_{LP} and BC_{MP} are near the start of the transmembrane helices LD (L173), and MD (M200), respectively. The Mg^{2+} ligands for BC_{LA} and BC_{MA} are in the helix connections C-D (L153 and M180, respectively). The BPh-*b*s are named BP_L and BP_M according to predominant interactions with subunit L or M. Each BPh-*b* binding site is a pocket formed mainly by non-polar residues from both subunits; there are no polar interactions between the protein and the tetrapyrrole nitrogens. BP_L (BP_M) is located near the centres of helices LC, LE (MC, ME) with its ring plane parallel to the axis of helix MD (LD). Accessory BChl-*b* and BPh-*b* are partially accessible from the environment.

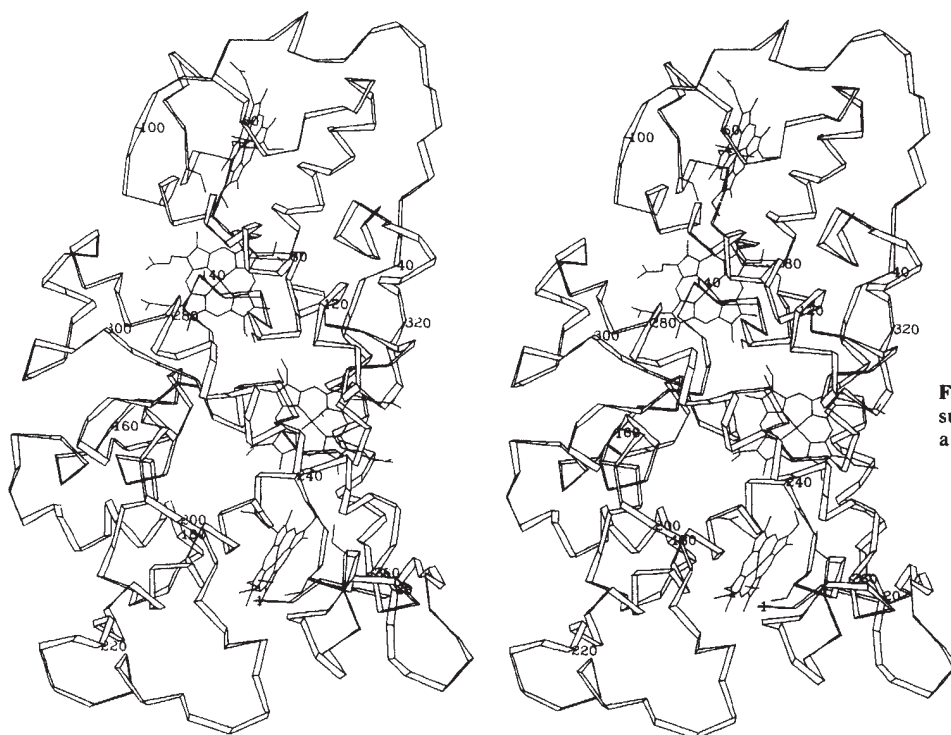


Fig. 5 Stereo drawing of the cytochrome subunit. The protein chain is represented as a ribbon; haem groups and linked cysteine side chains are shown as line drawings.

The non-haem iron is bound by five protein ligands, four histidines and one glutamic acid. The histidines are located in the transmembrane helices LD (L190), LE (L230), MD (M217) and ME (M264), and obey the L-M local 2-fold symmetry. They are arranged approximately in a plane perpendicular to the L-M local diad. The fifth iron ligand, Glu M232, sits on the local L-M diad. The structure near the non-haem iron is in good agreement with conclusions drawn from extended X-ray absorption fine-structure studies^{28,29}.

As reported previously¹⁰, we believe that the quinone identified in the electron-density map is the tightly bound quinone Q_A (menaquinone-9 in *Rps. viridis*³⁰). Q_A is located near BP_L ; its head group is bound exclusively to residues of M in the helix connection D-E with close contacts to His M217 (one of the iron ligands), to the peptide nitrogen of M258 and to Trp M250 whose side chain is nearly parallel to the Q_A head group. Its isoprenoid chain is mainly in contact with BP_L and with residues of L. It was shown that the loosely bound Q_B (ubiquinone-9 in *Rps. viridis*³⁰) can be lost during purification of the reaction centres¹⁴ or during crystallization³⁰. To find the Q_B site, the binding of the competitive inhibitors for Q_B *o*-phenanthroline³¹, terbutryn (a herbicide)³² and of ubiquinone-1 in the crystal was studied by soaking these compounds into the crystals (*o*-phenanthroline, 2 mM for 3 days; terbutryn, 1 mM for 3 days, followed by 0.01 mM for 3 days; ubiquinone-1, 1 mM for 3 days), collecting X-ray data and analysing these data using the difference-Fourier technique. These studies showed binding of the three compounds to a pocket formed near the H contact surface by parts of the transmembrane helices LD and LE, and by the LD-LE connecting segment. Additional binding sites were observed for *o*-phenanthroline (two), and ubiquinone-1 (seven). From these results we conclude that the single common binding site of the three compounds is the Q_B binding site. Only this site, which is related to the Q_A site by the L-M local diad, is close to the non-haem iron as required to explain the broadening of electron-spin resonance lines observed for Q_B^- (ref. 33). The Q_B site is in contact to the iron ligand His L190; the iron itself is located midway between Q_A and Q_B . This arrangement strongly suggests that the iron participates in electron transfer from Q_A to Q_B . However, experiments with iron-depleted reaction centres from *Rps. sphaeroides* showed that electron transfer is only moderately slower in the absence of the iron³⁴.

The binding sites of Q_A and Q_B are in regions of L and M, where both subunits markedly deviate from the L-M local symmetry. Whereas Q_A is completely shielded from the H subunit by the M chain, the probable Q_B binding site is accessible from H through an opening in the H contact surface. These observations correlate the different binding of Q_A and Q_B to structural differences between L and M; they also suggest that one function of H is related to Q_B binding. A more detailed description of the reaction centre structure near the prosthetic groups will be given elsewhere.

The H subunit

With 258 residues H is the smallest subunit of the reaction centre. The course of its polypeptide chain is shown in Fig. 4. The amino-terminus is near the cytochrome contact surface of L-M; residues H1, H2, H3 and H5 are in contact with residues from the cytochrome. Residues H12 to H37 form a helix which runs along the convex side of the surface formed by the transmembrane helices of M, with contacts to helices ME and MD (see Fig. 1). The amino-acid sequence in this helical region up to residue H32 is also primarily hydrophobic; the carboxy-end of the helix (H33-H37) and further on to H39 consists of charged residues (Arg-Arg-Glu-Asp-Arg-Arg-Glu) which interact partly among themselves, and probably also with the polar head groups of membrane lipids *in vivo*.

Altogether we find 11 transmembrane helices in the reaction centre complex, 6 running from the cytoplasmic side to the periplasmic side and 5 running in the opposite direction. The question of whether the dipole moments of these helices can facilitate electron transfer across the membrane, as proposed recently³⁵, requires a detailed inspection of the model and will be discussed elsewhere.

The absence of significant electron density for residues H48 to H56 near the end of the transmembrane helix indicates that this segment is disordered in the crystal. The segment H57-H115 lies on the surface of L-M; residues H66-H70 and H74-H78 form a two-stranded antiparallel β -sheet. The remainder of H is folded into a globular domain. Residues H156-H120 form two layers of β -sheet which enclose a hydrophobic core. Both sheets consist of three strands and show a right-handed twist. In sheet 1 the strands (H162-H174, H179-H187 and H191-H197) are antiparallel; in sheet 2 the middle strand (H207-H210)

is accompanied by an antiparallel strand (H200–H204) and a parallel strand (H156–H159). Near the carboxy-terminus residues H231–H249 form a helix that runs parallel to the H contact surface of L–M.

Little is known about the function of H in the reaction centre complex. Crosslinking studies¹⁶ indicate that H is close to the light-collecting complexes in the membrane; this observation suggests that H has a role in determining the relative orientation of light-collecting complexes and reaction centre. As mentioned above, H may also play a role in Q_B binding—residue H177 (Glu) and its neighbours have access to the Q_B binding pocket in subunit L. They could be involved in donating protons to the photochemically reduced Q_B . This is in accord with experimental results on L–M complexes of *Rps. sphaeroides*³⁶ which show abnormal electron transfer from Q_A to Q_B in the absence of H.

The cytochrome

The present model of the cytochrome subunit contains 333 residues. Figure 5 shows the course of the polypeptide chain and the location of the haem groups. Near the amino-terminus, residues C7–C11 and C20–C23 form an antiparallel β -sheet that lies on the L half of the cytochrome contact surface. From there the chain proceeds to the tip of the subunit (around C50); residues C25–C37 form a helix. In the following bulk of the subunit four recurring structural motifs can be observed: a helix runs parallel to the ring plane of each haem group (residues C67–C82, C102–C119, C222–C240 and C262–C286, respectively); the haems are covalently attached near the carboxy termini of these helices where the chains turn back towards the haems. The electron density strongly indicates that these covalent links are thioether bridges, and that the sequences of the haem binding segments are Cys–X–X–Cys–His as in other c-type cytochromes; the His residues are the fifth ligands to the haem irons. The haems are numbered according to the order of attachment to the protein; for haem 1 the first covalent link is to residue C87, haem 2, C132, haem 3, C244 and haem 4, C305. All haem irons appear to be six-coordinated; the sixth ligands to the irons of haem 1, 2 and 3 are located in the helices associated with the haems (residues C74, C110 and C233, respectively). The sixth ligand to the haem 4 iron (C124) is in the segment between the helix parallel to haem 2 and the haem 2 attachment site. According to the electron density the sixth ligands to the haem irons could be histidines or methionines.

As reported previously¹⁰, the haem groups are arranged with an approximate 2-fold symmetry: pyrrole ring atoms of haems 3 and 4 can be rotated onto those of haems 1 and 2 with polar angles $\phi = 117.2^\circ$, $\psi = -70.2^\circ$ and $\kappa = -176.5^\circ$ to an r.m.s. distance of 0.41 Å. Haems 1 and 2 and haems 3 and 4 are related by improper rotations ($\sim 105^\circ$) with large screw components (~ 12 Å). Using the same method as for the L–M comparison²³, 66 C^α positions from the region C226–C315 could be rotated onto corresponding C^α positions from between C67 and C142 to an r.m.s. distance of 1.1 Å. The polar angles of this rotation are $\phi = 116.7^\circ$, $\psi = -68.9^\circ$ and $\kappa = -177.8^\circ$, very close to those for the haem rotation. This internal 2-fold symmetry includes about one third of the cytochrome subunit. More extended structural homologies with 2-fold symmetry in protein molecules have been reported for rhodanese³⁷ and γ -crystallin³⁸. The intramolecular symmetry is broken in the segment connecting the binding sites of haem 2 and 3; this segment contributes to the contact of cytochrome to L–M. The carboxy-terminal segment ends near the helix associated with haem 2. Haem 1 is accessible from the environment at its pyrrole rings I and IV; haem 2 appears to be almost completely buried; and haems 3 and 4 are accessible at their propionic acid groups. Pyrrole rings I and IV of haem 3 are closest to the special pair of BChl-*b* in the L–M complex. Apparently, protein folding in the cytochrome subunit of the reaction centre is different from both cytochromes C2 and cytochromes C3 with known three-dimensional structure (see ref. 39 for review).

Structure of related proteins

Reaction centres from other purple bacteria. Amino-acid sequences are known for the reaction centre subunits L, M and H from *Rps. capsulata*⁴⁰, and for L and M from *Rps. sphaeroides*^{41,42}. The sequences of L and M from *Rps. viridis* show between 50 and 60% homology with those of the corresponding subunits from the other two bacteria. On the basis of hydrophobicity five transmembrane helices each were postulated^{40–42} in segments of L and M homologous with the transmembrane helix regions in our model. All the ligands to the BChl and to the non-haem iron described above are also conserved, and the polypeptide segments connecting these ligands have the same length in all three organisms. These observations clearly indicate that the structures of L and M from *Rps. capsulata* and *Rps. sphaeroides*, and the arrangement of BChls and non-haem iron are very similar to those found for *Rps. viridis*. Significant differences in polypeptide chain length occur exclusively at the carboxy-termini where L from *Rps. viridis* is shorter by 8 residues, and M is longer by 17 residues. These additional residues of M are in contact with the cytochrome in *Rps. viridis*: the reaction centres of *Rps. capsulata* and *Rps. sphaeroides* lack a permanently bound cytochrome subunit.

For the H-subunits from *Rps. capsulata* and *Rps. viridis* the sequence homology is 37% (ref. 21). Common features of these two H-subunits include a hydrophobic region near the amino terminus which corresponds to the transmembrane helix of H in our model. In addition, many of the residues involved in the contact between H and L–M are conserved in both organisms, so that structural similarity can also be expected between the H subunits from *Rps. capsulata* and *Rps. viridis*.

Photosystem II. Recently, amino-acid sequences of polypeptides from photosystem II reaction centres^{43–47} have been reported. Sequence homologies were found between L and M of purple bacteria and the D1 polypeptide (also referred to as Q_B protein or herbicide binding polypeptide) from photosystem II of higher plants^{40,42} and between D1 and D2 (ref. 45) which is also a constituent of photosystem II. In these plant proteins, histidines which correspond to the ligands of the special pair, and to those of the non-haem iron in our model are conserved. Even a Trp residue corresponding to M250, which forms part of the Q_A binding site in reaction centres of purple bacteria, can be found in the D2 proteins. These observations corroborate our recent proposal⁴⁸ that proteins D1 and D2 form the core of the photosystem II reaction centre, similar to the subunits L and M in purple bacteria. Based on functional and sequence homology we believe that D1 and D2 span the membrane five times, contrary to a proposed seven-helix structure⁴⁹, where the helix connections A–B and D–E were probably misinterpreted as transmembrane helices.

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Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*

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We show here that Agrobacterium tumefaciens virulence (Vir) gene expression is activated specifically by the plant molecules acetosyringone (AS) and α -hydroxyacetosyringone (OH-AS). These molecules induce the entire vir regulon in Agrobacterium as well as the formation of T-DNA intermediate molecules. AS and OH-AS occur specifically in exudates of wounded and metabolically active plant cells and probably allow Agrobacterium to recognize susceptible cells in nature.

SOIL bacteria often form specialized interactions with plant cells. Usually a particular bacterium interacts with only a few species and/or types of plant cells^{1,2}. A primary step in the formation of a bacterial/plant interaction is the detection by the bacterium of the appropriate susceptible plant cell. This recognition then triggers the activation of the bacterial genes whose products direct the development and/or maintenance of the interaction. The soil is a complex biological and chemical environment, and the signals that mediate the detection of a specific plant cell by a bacterium are unknown.

A. tumefaciens, a soil phytopathogen, genetically transforms dicotyledonous plant cells to cause the neoplastic disease crown gall^{3,4}. Only cells that have been wounded are seen to be susceptible^{5,6}. The bacterium transfers a specific segment of DNA, the T-DNA, from its large (> 200 kilobases, kb) tumour-inducing (Ti) plasmid to the susceptible plant cell, where it becomes integrated into the nuclear genome^{7,8}. In the Ti plasmid, the T-DNA is defined and bounded by identical 25-base pair (bp) direct repeats; only DNA between these T-DNA borders is transferred to the plant genome⁹⁻¹². During co-cultivation with plant cells, independent T-DNA circles are formed in *Agrobacterium*; these molecules arise by a specific recombination between the 25-bp sequences at the ends of the T-DNA and are potential intermediates in the transfer of the T-DNA from *Agrobacterium* to the plant cell¹³.

The Ti plasmid genes required for plant transformation are not contained in the T-DNA but are located in the ~40-kb *vir* region¹⁴⁻¹⁶. Genetic analysis of the *vir* region of the A6 Ti plasmid has shown that it encodes at least six separate complementation groups, *virA*, *B*, *C*, *D*, *E* and *G*, and *pinF* that are organized as a single regulon (ref. 17; S.E.S., in preparation). In the vegetative bacterium only *virA* and *virG*, the *vir* regulatory genes, are significantly expressed; however, when *Agrobacterium* is co-cultivated with plant cells the expression of *virB*, *C*, *D*, *E*,

G and *pinF* is induced to high levels^{17,18}. This activation of *vir* expression by plant cells probably initiates the steps of T-DNA transfer and integration into the plant cell genome. We have shown that *vir* induction and the production of T-DNA circles is mediated by a small diffusible factor produced by actively growing plant cells¹⁷. Here, we purify and establish the chemical identity of this factor, and demonstrate that its production is related to plant cell wounding and that its recognition by the bacterium is a highly specific process. This factor is likely to be the signal that allows *Agrobacterium* to recognize in nature a plant cell susceptible to transformation.

Purification of signal molecules

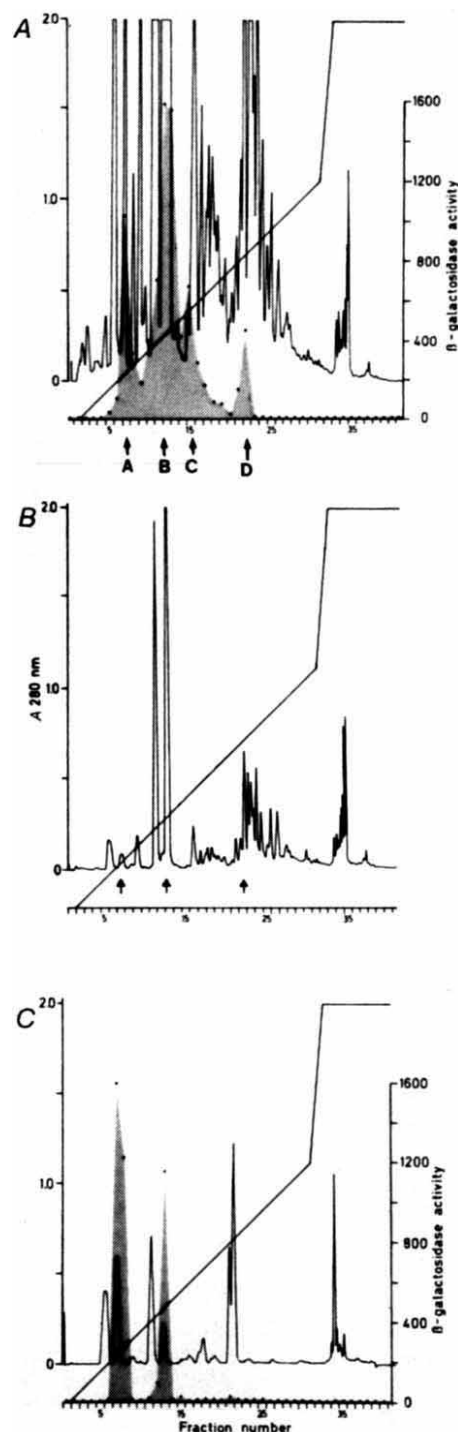
To purify the plant molecule(s) that specifically signals *Agrobacterium* to initiate its interaction with plant cells requires first, an efficient and quantitative bioassay for a primary event in this transformation process, specifically the induction of *vir* gene expression; and second, a starting source of the signal molecule(s). We have previously described an assay for *vir* induction in *Agrobacterium* that used gene fusions between the pTiA6 *vir* loci and the *Escherichia coli lacZ* gene^{17,18}. Briefly, in a bacterium carrying a *vir::lac* gene fusion, the production of β -galactosidase (the *lacZ* gene product) is controlled by the *vir* locus to which *lacZ* has been fused. Thus, the state of expression of the locus can be monitored by measuring the β -galactosidase activity present in the bacterium; increased activity indicates increased *vir* expression. (The relative amount of induced activity reflects the relative amount of *vir*-inducing activity to which the cell has been exposed.) Here we use *Agrobacterium* strain A348(pSM30) (ref. 17) to detect and measure *vir*-inducing activity. This strain contains wild-type pTiA6 and pSM30, a *virB::lac* fusion plasmid, and gives high levels (up to ~100-fold of basal activity) of induced β -galactosidase activity¹⁷.

Fig. 1 RPC/FPLC fractionation of the *vir*-inducing activity in plant-cell exudates. The organic compounds present in plant cell medium conditioned with root culture (*cmr*; A, B), or with leaf disks (*cml*; C), were prepared by chloroform extraction and analysed by RPC/FPLC. The material analysed in A, B and C was prepared from 2 litres *cmr*, 40 ml *cmr* and 50 ml *cml*, respectively. Each sample was fractionated on a C-2/C-18 RPC column eluted with a linear methanol gradient. In A and C, column fractions were bioassayed for *vir*-inducing activity in *Agrobacterium*. The diagonal line represents the elution gradient and the solid line indicates the ultraviolet absorbance measured at 280 nm of the column fractions; shaded curves indicate relative specific units of β -galactosidase activity induced in the *virB::lacZ* tester strain A348(pM30) by the column fractions. The major peaks of *vir*-inducing activity in A are indicated by arrows labelled A, B, C and D. B, Arrows indicate peaks corresponding to peaks A, B and D of A.

Methods. Root culture of *N. tabacum* transformed with *Agrobacterium rhizogenes* A15834 was grown and maintained as described previously¹⁷. Transformed roots were used because they are easy to propagate. Every 72 h the conditioned medium (*cmr*) was removed and stored at -20°C . Leaf disks were prepared from 6-week-old untransformed *N. tabacum* SR1 plants, and 2-g samples of 1.5-cm-diameter disks were incubated in 50 ml MS medium (Murashige and Skoog²⁸ salts, 3% sucrose, supplemented with 0.018% K_2HPO_4 , 0.01% inositol, 0.0001% biotin, pH 5.5) in 150-mm Petri dishes. After 72 h, the conditioned medium (*cml*) was removed and stored at -20°C . The conditioned medium (*cmr* and *cml*) was filtered through 0.22 μm nitrocellulose and the filtrate extracted twice with a 25% volume of chloroform and the pooled chloroform phase was back-extracted with 1 vol. MS medium, rotary-evaporated to dryness and resuspended in 500 μl 20% CH_3OH , 0.1% CH_3COOH for analysis by RPC/FPLC. (Note that initial solvent extraction experiments were performed to determine the solvent partitioning character of the *cmr* and *cml* *vir*-inducing activities. In these experiments 50 ml of the respective conditioned medium was extracted with chloroform; the interphase and chloroform phase were lyophilized and each resuspended in 2 ml MSSP (MS medium supplemented with 12.5 mM sodium phosphate, pH 5.5¹⁷), and the aqueous phase was blown with a stream of air to remove traces of chloroform. Each of these samples was bioassayed for *vir*-inducing activity; only the chloroform phase material contained this activity.) A Pep RPC pre-packed 5 mm $\times$ 50 mm column (HR5/5) containing 6- μm silica particles with C-2 and C-18 alkyl side chains (Pharmacia) was pre-equilibrated with 10% CH_3OH , 0.1% CH_3COOH . The column was run at a flow rate of 0.7 ml min^{-1} using a Pharmacia FPLC system equipped with the LCC500 chromatographic programmer. A single-path ultraviolet monitor was used to monitor absorbance at 280 nm. The column was eluted with a linear gradient of 10–60% CH_3OH : H_2O (v/v), 0.1% CH_3COOH . We collected 40 1.4-ml (2-min) fractions; 140- μl aliquots (A) or the entire fractions (C) were lyophilized, resuspended in 1.5 ml MSSP and bioassayed for *vir*-inducing activity. Overnight cultures of strain A348(pSM30) were centrifuged and resuspended in MSSP medium. Material to be tested for *vir*-inducing activity was inoculated with bacteria at 0.1 absorbance unit ml^{-1} at 600 nm cm^{-1} . Incubations were for 10 h at 28°C and 200 r.p.m. Specific units of β -galactosidase activity were determined as described previously^{17,18} and are expressed as U per bacterial cell.

Induction of *vir* expression occurs during co-cultivation of *Agrobacterium* with plant cells, and during incubation of bacteria in plant-cell exudates. We have shown that the medium in which *Nicotiana tabacum* root culture has grown (designated *cmr* for conditioned medium roots) contains substantial amounts of a *vir*-inducing activity¹⁷. This activity stimulates the expression of each of the inducible pTiA6 *vir* loci, and also the formation of T-DNA circular intermediates, indicating that it triggers in *Agrobacterium* the initiation of plant cell transformation. This activity has relative molecular mass less than 1,000; is stable to boiling, freezing, lyophilization, and high and low pH; and is partially hydrophobic, as it is retained by silica C-18 and completely elutes from this matrix with 40% CH_3OH (ref. 17). Here we purify and identify this *cmr* activity.

The above properties suggest that the *cmr* *vir*-inducing activity is composed of one or more small organic molecules; that it completely partitions into the organic solvent chloroform (Fig. 1) confirms this identity, and provided a basis for its purification. The *cmr* *vir*-inducing activity was fractionated by reverse-phase chromatography (RPC) using a high-resolution fast-performance liquid chromatography (FPLC) system. Activity was localized to two major and two minor peaks that had eluted, respectively, with 18 (peak A), 27.5 (peak B), 32



(peak C) and 42% (peak D) CH_3OH (Fig. 1A). Thus, the *cmr* *vir*-inducing activity is represented by at least four distinct compounds. When less material is loaded onto the FPLC column (Fig. 1B), peak B is the major component of the organic fraction of *cmr*. Because of their low abundance, the peak C and peak D activities were not analysed further. The signal molecules in peaks A and B were purified to homogeneity by further RPC fractionation and analysed by gas chromatography/mass spectroscopy (GC-MS) (Fig. 2) and ultraviolet absorption spectroscopy (Fig. 3).

Identification of *vir* inducers

The peak B compound, the major *cmr* *vir*-inducing molecule, is 4-acetyl-2,6-dimethoxyphenol, based on several experimental observations: (1) The GC/MS spectrum of the peak B compound

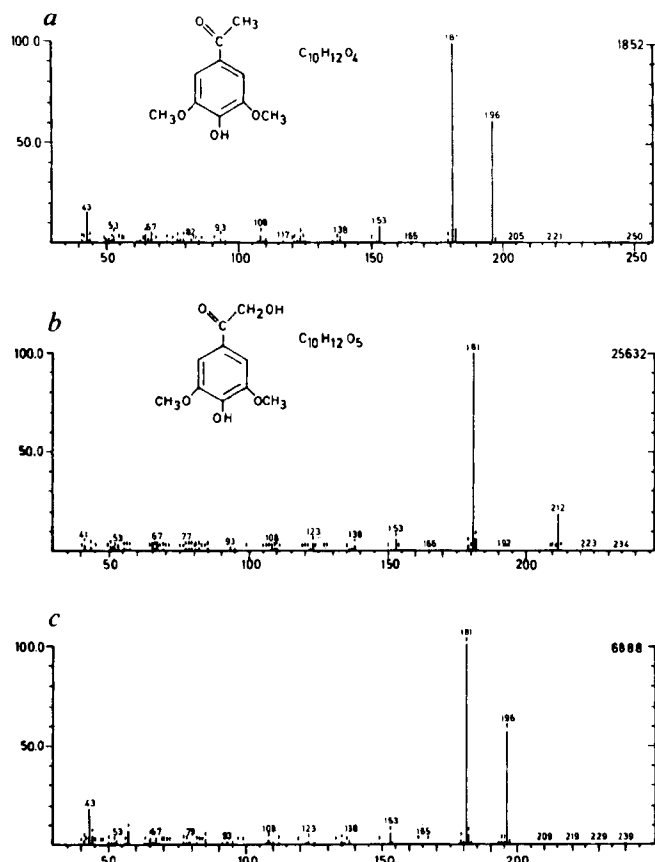


Fig. 2 Mass spectra of *vir*-inducing molecules. The mass spectra of purified peak B compound (Fig. 1), purified peak A compound (Fig. 1) and authentic acetosyringone (Janssen Chimica), are shown in *a*, *b* and *c*, respectively. Vertical axes, relative intensity of the fragment ions; horizontal axes, the mass/charge ratio of the fragment ions. The determined chemical formulae and structures of peak B and peak A (from Fig. 1) are indicated in *a* and *b*, respectively.

Methods. The biologically active compounds present in the peak A and B fractions were purified to homogeneity by RPC/FPLC; fractionation conditions were identical to those described in Fig. 1, except for the column solvent. The fractions containing peak A or peak B (from Fig. 1A) were pooled, lyophilized, resuspended in 5% acetonitrile, 0.1% trifluoroacetic acid (TFA) and injected onto the C-2/C-18 column equilibrated with this buffer. The column was eluted with a linear gradient of 5–30% acetonitrile, 0.1% TFA, and monitored for ultraviolet absorbance at 280 nm and for biological activity. For each sample a single peak was resolved from other minor ultraviolet-absorbing compounds, and a portion of the peak fraction was lyophilized, dissolved in 500 μ l chloroform and evaporated to 40 μ l under a nitrogen stream for GC/MS analysis. Separate portions of each sample were dissolved in CH_3OH for ultraviolet absorption analysis (Fig. 3) and in MSSP for analysis of biological activity. We injected 0.5 μ l of sample into a 0.32 mm $\times$ 25 m Carlo Erba (HRGC) gas chromatography column SF-52, directly coupled to a Finnigan 4000 mass spectrometer. GC fractionation was with a 50–250 $^\circ$ gradient, 5 $^\circ$ min $^{-1}$. Data were collected and processed on a Nova 3 computer (Data General).

(Fig. 2a) indicates a molecule of relative molecular mass (M_r) 196, chemical composition $\text{C}_{10}\text{H}_{12}\text{O}_4$ and general structure acetyl-dimethoxyphenol. (2) Comparison of the ultraviolet absorption spectrum of the peak B compound in CH_3OH with its spectrum in $\text{CH}_3\text{OH}/\text{NaOH}$ (Fig. 3a) indicates that the molecule exhibits a strong absorption shift to longer wavelengths in the presence of NaOH ; this base-induced redshift is diagnostic of phenolic compounds in which the phenolic hydroxyl group is *para* (but not *ortho* or *meta*) to a conjugated ring substituent, such as a ketone or allyl group¹⁹. (3) The *p*-hydroxyl derivative, 4-acetyl-2,6-dimethoxyphenol, commonly termed acetosyringone (AS), is commercially available and greatly stimulates *vir*

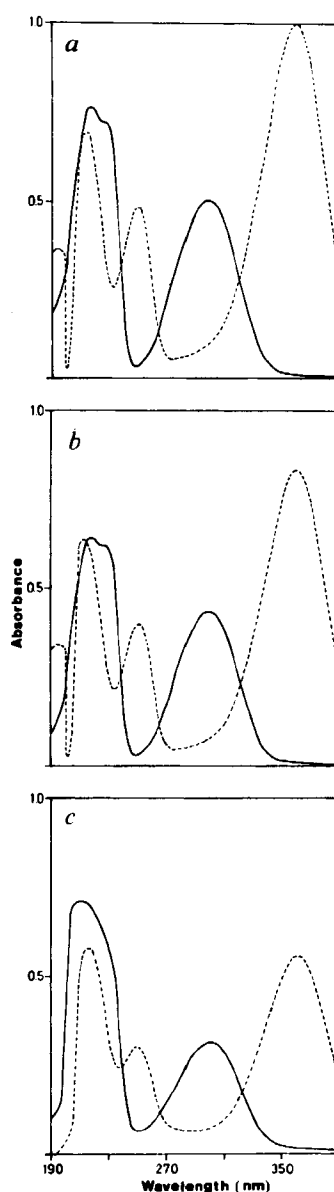


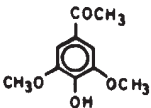
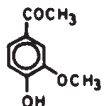
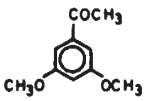
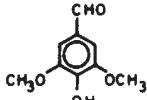
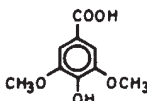
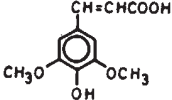
Fig. 3 Ultraviolet absorption spectroscopy of *vir*-inducing compounds purified from *cmr*. Spectra were determined in both CH_3OH (solid line) and $\text{CH}_3\text{OH}/\text{NaOH}$ (dashed line). *a*, Spectra of peak B compound; *b*, spectra of commercially obtained acetosyringone; *c*, spectra of peak A compound.

Methods. Absorption spectra were measured in a Beckman DU-6 spectrophotometer scanning at 60 nm min $^{-1}$. For each compound dried material was resuspended in CH_3OH (HPLC grade, Rathburn Chemicals) to an approximate concentration of 50 μM , and scanned against CH_3OH as a blank solution. The pH of the sample was subsequently adjusted to basic pH by the addition of 1 M NaOH to 20 mM and the sample scanned against $\text{CH}_3\text{OH}/20$ mM NaOH. (Note that when the base-adjusted sample of each compound is readjusted to acid pH by adding 1 M HCl to 40 mM, the determined spectrum is equivalent to the original CH_3OH spectrum of the compound (data not shown). Thus, the base-induced redshift of each compound is fully reversible in the conditions used.)

expression in *Agrobacterium* (see below). AS and the purified peak B compound co-elute on RPC/FPLC (data not shown) and their GC/MS spectra are indistinguishable (Fig. 2). Furthermore, their respective ultraviolet spectra, both in CH_3OH and in $\text{CH}_3\text{OH}/\text{NaOH}$, are identical (Fig. 3a, b). (4) The quantitative *vir*-inducing activities of authentic AS and the peak B compound are equivalent. In the experiment described in Fig. 4, the units of induced β -galactosidase activity as a function of concentration of inducing compound were determined for each compound at several concentrations; the respective activity/concentration curves are identical.

The peak A compound, the second major *cmr* *vir*-inducer, is 4-(2-hydroxyacetyl)-2,6-dimethoxyphenol, termed α -hydroxy acetosyringone (OH-AS), an analogue of AS. This identification is based on the following observations: (1) The GC/MS spectrum of the peak A compound (Fig. 2c) indicates a molecule of M_r 212 whose only significant fragmentation product has M_r 181. The GC/MS spectrum of compound A following trimethylsilylation indicates a mass increase corresponding to two trimethylsilyl groups (data not shown). Thus, compound A contains two hydroxyl groups and has general structure of either (3-propanol)dimethoxyphenol ($\text{C}_{11}\text{H}_{14}\text{O}_4$) or (2-hydroxyacetyl)dimethoxyphenol ($\text{C}_{10}\text{H}_{12}\text{O}_5$). Only the latter structure is consistent with the observation that the peak A compound is more polar than AS, as it elutes before AS on RPC/FPLC

Table 1 Biological activity of derivatives of acetosyringone

<i>a</i> acetosyringone		110	109	93	29
<i>b</i> acetovanillone		80	33	1.9	1.6
<i>c</i> 3,5-dimethoxyacetophenone		ND	1.0	ND	ND
<i>d</i> 4-hydroxyacetophenone		ND	1.1	ND	ND
<i>e</i> syringaldehyde		86	51	2.2	ND
<i>f</i> syringic acid		21	8.6	1.7	ND
<i>g</i> sinapinic acid		104	98	68	18

Acetosyringone (*a*) and six related compounds; acetovanillone (*b*); 3,5-dimethoxyacetophenone (*c*); 4-hydroxyacetophenone (*d*); syringaldehyde (*e*); syringic acid (*f*); and sinapinic acid (*g*), were tested for their ability to induce β -galactosidase in the *Agrobacterium virB::lacZ* strain A348(pSM30). All compounds were purchased from Janssen Chimica, and prepared as 0.1 M solutions in dimethyl sulphoxide. Each compound was serially diluted into MSSP (Fig. 1, legend) medium to 200, 50, 5 and 0.5 μ M (*a, b, e, f, g*) or 50 μ M (*c, d*). These solutions were inoculated with bacteria at 0.05 absorbance unit ml^{-1} at 600 nm cm^{-1} and incubated at 28 °C, 200 r.p.m. After 12 h, the bacterial β -galactosidase activity in each sample was determined. The data are expressed as activity of the bacteria incubated in the presence of a compound relative to the basal activity present in bacteria incubated without added compound. The basal activity for the pSM30 strain is 10 U. ND, not determined.

(Fig. 1). (2) Comparison of the ultraviolet adsorption spectrum of the peak A compound in CH_3OH with its spectrum in $\text{CH}_3\text{OH}/\text{NaOH}$ indicates that, similarly to AS, this molecule exhibits a strong base-induced redshift (Fig. 3c). Thus, compound A contains a phenolic hydroxyl group *para* to a conjugated ring substituent. Furthermore, the absorption spectra of compound A are almost identical to the equivalent spectra of AS; the respective absorption maxima occur at identical wavelengths (the largest maxima occur at 298 nm in CH_3OH and 355 nm in $\text{CH}_3\text{OH}/\text{NaOH}$), although their relative extinction coefficients are different. These similarities strongly indicate that the peak A compound is very closely related to AS. (3) The quantitative *vir*-inducing activity of the peak A compound is approximately equivalent to that of AS (Fig. 1). As described below, the structure/activity specificity of *vir*-induction indicates that only molecules with similar structure to AS induce *vir* expression to high levels (Table 1).

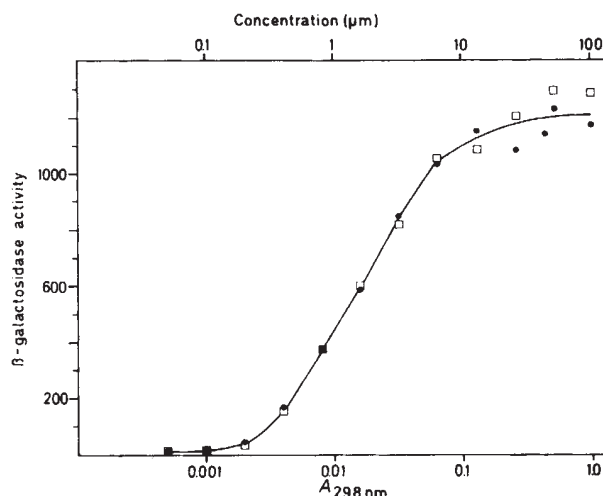


Fig. 4 Comparison of *vir*-inducing activity of purified peak B compound (Fig. 2) and commercially obtained acetosyringone. Vertical axis, β -galactosidase activity (U bacterium $^{-1}$) in the *Agrobacterium virB::lacZ* strain A348(pSM30); lower horizontal axis, relative concentration of purified peak B compound (Fig. 2) or authentic AS (Janssen Chimica) in the inducing medium, measured as absorbance at 298 nm (cm^{-1}). The extinction coefficient of AS at 298 nm is 10,300 (ref. 29); this value was used to calculate the concentration of inducer compound in the inducing medium, indicated by the upper horizontal axis.

Methods. Purified peak B compound and authentic AS were separately dissolved in MSSP to 1.0 absorbance unit ml^{-1} at 298 nm (cm^{-1}), measured against fresh MSSP. Each solution was serially diluted and the resultant samples were inoculated with bacterium at 0.09 absorbance unit ml^{-1} at 600 nm (cm^{-1}). Samples were incubated for 14 h at 28 °C, 200 r.p.m. and the β -galactosidase activity of the bacteria in each sample determined.

Thus, we have identified plant molecules that induce *vir* expression. Our next experiments seek to provide insight into the relationship between these molecules and *Agrobacterium* in nature. For the *Agrobacterium*/plant transformation system to be most efficient, its activation should be limited to the presence of susceptible plant cells. This could be achieved if activation is signalled only by molecules specific to these cells. For instance, such molecules should first, be produced by different types of plant cells, as *Agrobacterium* can transform several different cell types²⁰; second, be specifically synthesized by susceptible plant cells, such as wounded cells; and third, be available to the bacterium in quantities sufficient for efficient activation. The following experiments demonstrate that AS has these properties and that activation of *Agrobacterium* is a highly specific process.

Different plant tissues produce AS/OH-AS

To determine whether AS and OH-AS are specific to roots or whether they are also produced by other plant tissues, we purified and identified the *vir*-inducing activity produced by leaf cells (Fig. 1). Medium in which *N. tabacum* leaves that have been cut into disks have been cultured (*cml*; conditioned medium leaves) contains substantial amounts of inducing activity that completely partitions into chloroform. The RPC/FPLC profile and corresponding bioassay profile of this material (Fig. 1C) show that the *cml* activity fractionates into two peaks that co-elute with OH-AS and AS. Subsequent purification and GC/MS and ultraviolet spectrophotometric analysis of the active molecules in these peaks confirmed that the major *vir*-inducing activity in *cml* is composed of OH-AS and AS (data not shown). Thus, these two compounds are present in the exudates of at least two different plant tissues.

AS and OH-AS are exudate-specific

In the soil, *Agrobacterium* probably detects plant cells through their exudates. To determine whether AS and OH-AS are

exudate-specific we assessed their relative concentrations within the *N. tabacum* leaf disks of Fig. 1C. These disks were extracted with chloroform and the extract was analysed by RPC/FPLC fractionation and corresponding bioassay. The relative concentrations of AS and OH-AS are <0.5% of the organic compounds present in the total leaf disks (data not shown). In comparison, the relative concentrations of these compounds in the *cml* extract is ~25% and greater (Figs 1C, 5A). Thus, AS and OH-AS probably do not leak out of damaged plant cells and are exudate-specific compounds.

Concentration of *vir* inducers in *cmr/cml*

Figure 4 shows the relationship between the concentration of AS (both commercial and *cmr*-purified) and induction of *virB* expression as measured in units of β -galactosidase activity. Under the conditions used, AS at $\geq 10 \mu\text{M}$, and AS at $1.5 \mu\text{M}$, induce maximal (1,200 U) and half-maximal (600 U) expression, respectively. Note that AS induction does not significantly affect cell viability (that is, induction is a non-lethal event), and that concentrations of AS $>200 \mu\text{M}$ are not significantly toxic to the bacteria. Using the dose-response curve of Fig. 4, the concentration of *vir*-inducing compounds in plant-cell exudates relative to AS can be estimated. Our starting *cmr* and *cml* preparations typically induce between 250 and 500 U β -galactosidase activity in A348 (pSM30); this activity approximately corresponds to between 0.5 and $1 \mu\text{M}$ AS.

Biological activity of acetylsyringone

The primary molecular signal for the initiation in *Agrobacterium* of the events of plant cell transformation should induce in the bacterium both the entire *vir* regulon and the initial steps of T-DNA transfer. We determined that AS has these activities. Several *vir::lac* and *pin::lac* *Agrobacterium* strains were incubated in $20 \mu\text{M}$ AS (both commercial and *cmr*-purified) and assayed for β -galactosidase activity. Inductions of all the previously identified inducible *vir* loci (B, C, D, E, G) and *pinF* was obtained, and the levels of induction were at least 20–50% higher than those stimulated during co-cultivation with plant cell cultures or incubation with *cmr*¹⁷ (data not shown). AS also induces the production of T-DNA intermediates: when bacteria are incubated with $20 \mu\text{M}$ AS for 12–18 h, T-DNA intermediates¹³ are found at a frequency two- to fivefold greater than that obtained following 48-h co-cultivation with protoplasts¹⁷ (data not shown). We have also seen that AS induces the appearance and/or disappearance of several major proteins in *Agrobacterium* (P. Engström, P.Z. and S.E.S., in preparation). Thus, a single compound is sufficient to trigger the complete activation of *vir* and the initial events of T-DNA transfer; and AS (probably OH-AS also) is a primary signal for plant cell transformation by *Agrobacterium*.

Molecular specificity of *vir* induction

Table 1 shows the respective *vir*-inducing activities of AS (a) and analogues of AS (b–g). These results indicate that *Agrobacterium vir* expression is efficiently induced by molecules that conform to a structure best represented by AS itself, and suggest that *Agrobacterium* has evolved to recognize and respond to AS as a specific signal for the activation of plant cell transformation. For instance, AS without one methoxy group (b) has greatly attenuated *vir*-inducing activity, whereas AS without both methoxy groups (d) or its hydroxyl group (c) is inactive. The acetyl substituent of AS is important for activity. The formyl (e) and carboxylic acid (f) analogues of AS have attenuated activity, whereas the cinnamic acid analogue of AS, sinapinic acid (g), has approximately equivalent activity to AS. This latter result is interesting in that sinapinic acid is a precursor of lignin, an integral cell-wall constituent of all vascular plants (see below).

Wounding stimulates AS/OH-AS production

Although many dicotyledonous plant cells can be transformed by *Agrobacterium*, only cells that have been wounded or traumatized

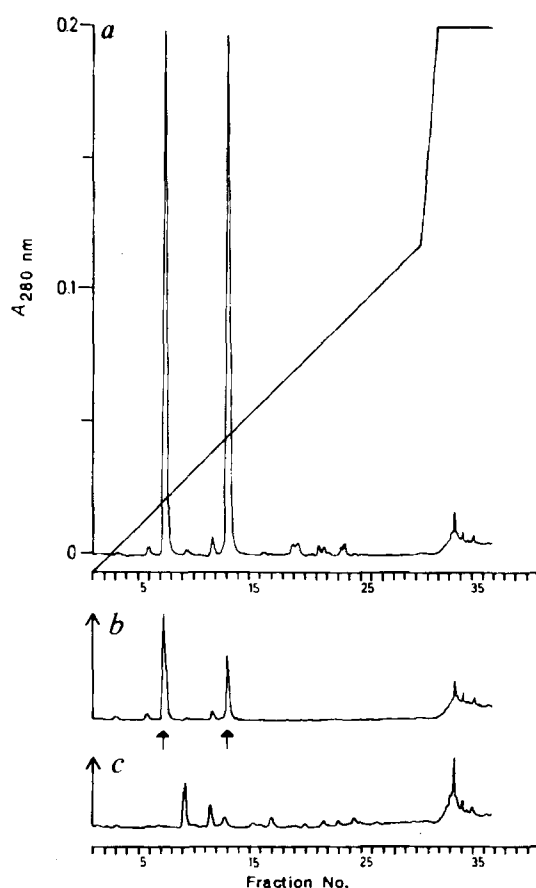


Fig. 5 Effect of wounding and inhibition of plant cell metabolism on production of AS and OH-AS by plant cells. RPC/FPLC fractionations of equivalent amounts of medium conditioned with: a, 1.5-cm-diameter leaf disks; b, intact leaves; c, leaf disks in the presence of cycloheximide. The vertical axes are drawn to the same scale in a–c. Arrows, elution positions of OH-AS (fraction 7), and AS (fraction 13).

Methods. Leaves (4 g) excised from 6-week-old *N. tabacum* SR1 plants were treated as described below and incubated in 50 ml MS medium in a 150-ml Petri dish. Care was taken to use visually equivalent leaves in each experiment. a, 5–6 1-cm² disks were obtained per leaf; b, intact leaves were used; c, same as a but with 5 p.p.m. (12.8 μM) cycloheximide (Actidion, 99%; Aldrich Chemicals) added to the medium. After a 72-h incubation, 40 ml of each respective conditioned medium was recovered, filtered through 0.22 μm nitrocellulose and 35 ml extracted with chloroform (Fig. 1), and the organic pellet was resuspended in 400 μl 10% CH_3OH , 0.1% CH_3COOH . We analysed 100 μl of the sample by RPC/FPLC using the conditions described in Fig. 1. The remaining 5 ml of conditioned medium was bioassayed for *vir*-inducing activity; the units of β -galactosidase activity induced by the conditioned media of a, b and c were 565, 135 and 20, respectively.

ized are susceptible. Therefore, we tested whether the production of AS and OH-AS is stimulated by plant cell wounding. Figure 5 shows the RPC/FPLC profiles of the organic extracts of *cml* produced by equivalent amounts of *N. tabacum* leaf disks (wounded cells; Fig. 5a) or intact leaves (unwounded; Fig. 5b). Comparison of these profiles indicates that the 'wounded' *cml* contains >10-fold more AS and OH-AS than the 'unwounded' *cml*, demonstrating that wounding stimulates the appearance of these molecules in the cell exudate. The low levels of AS and OH-AS in the 'unwounded' leaf exudate could be caused by the cut stem surfaces of the leaves.

These results do not define which cells of the wounded tissue produce AS and OH-AS. For example, damaged or dead cells could release AS, although such cells are not good targets for

Agrobacterium infection. We tested whether active plant cell metabolism is required for the production of AS by analysing the *cml* produced by leaf disks incubated in cycloheximide. This material does not contain AS and OH-AS (Fig. 5c). These results confirm and extend our previous observation that in plant/*Agrobacterium* co-cultivations only actively growing plant cell cultures are able to stimulate efficient *vir* gene expression¹⁷. Our data concerning the production of AS and OH-AS in total suggest that these compounds are specifically synthesized and exuded by metabolically active wounded cells.

Discussion

The molecules that we have purified signal *Agrobacterium* to activate the expression of its virulence genes, setting in motion a series of molecular events ultimately resulting in the transfer of T-DNA from the bacterium to the plant cell.

Wounded cells are known to be susceptible to *Agrobacterium* infection, perhaps because the intact cell walls of undamaged cells restrict T-DNA transfer, or because T-DNA integration is dependent on host-cell replication and wounding stimulates this replication. Because *Agrobacterium* responds to AS and OH-AS to initiate plant cell transformation, these molecules potentially represent the signal that *Agrobacterium* detects and recognizes in the soil as susceptible plant cells. The presence of AS and OH-AS specifically in the exudates of wounded but actively metabolizing plant tissues supports our hypothesis.

The activation of *Agrobacterium vir* expression by plant signal molecules probably involves at least two steps: extracellular recognition and intracellular response. The first step could depend on the signal molecule acting as a chemical attractant and/or nutritive source for the bacterium. The latter must depend on the ability of the bacterium to convey the information of the signal from outside to inside the cell and to activate *vir* expression. The mechanism of these events is unknown. Induction of the pTiA6 *vir* region is attenuated in *virA* and does not occur in *virG* mutant bacteria (S.E.S., in preparation). Also, the amino-acid sequence of the *virG* gene product is highly homologous with several positive regulatory proteins of *E. coli*²¹. Because AS can cause the induction of the complete *vir* regulon, we suggest that this compound acts to activate allosterically the *virG* protein, which then activates *vir* transcription by directly interacting with *vir* gene promoter sequences. The *virA* protein potentially functions in the initial extracellular/intracellular recognition and/or intracellular transport of the signal molecule.

AS and OH-AS have not previously been identified as natural component of plant cells, suggesting that the appearance of these molecules in nature is not widespread. Thus, these molecules potentially represent to *Agrobacterium* only those cells which are its desired targets. In addition, these or related molecules might also serve to initiate other bacterial/plant interactions in the soil. The observed resistance of most monocotyledonous plants to *Agrobacterium* could result because these

plants do not produce, or only produce in low quantities, *vir* signal molecules. AS and OH-AS could be useful for obtaining *Agrobacterium* transformation of plant species previously seen to be resistant to *Agrobacterium*, and also for the analysis of the initial molecular steps of the T-DNA transfer process.

Although it is important for *Agrobacterium* to detect and respond to such molecules, it is equally important that it does not respond to closely related but functionally different molecules; we have shown that *vir* induction is most efficiently stimulated by AS and by the lignin precursor sinapinic acid. This latter compound is not present in detectable quantities in our exudate preparations; however, sinapinic acid could be present in the soil in exudates of wounded cells in the process of cell-wall rebuilding.

It is interesting to speculate on the function of AS and OH-AS in plants. These compounds are likely products of the shikimic acid biosynthetic pathway that provides the plant cell with the precursors to a broad spectrum of molecules, including the flavonoids, and lignins²²⁻²⁴. These classes of compounds are important to a plant subjected to stress or injury. For example, many flavonoid-derived phytoalexins are potent inhibitors of the growth of invading pathogens²⁵, whereas lignin, a major component of the cell wall, provides a physical barrier to invasion²⁶. Thus, AS and OH-AS could be part of the wound response²⁷ of plant cells. These compounds are potentially toxic to other soil pathogens, and *Agrobacterium* has evolved to be resistant to AS and OH-AS and to use these chemicals to recognize wounded cells. Alternatively, the compounds could be products related to lignin repair in damaged cells.

We propose that there is a continual excretion of wound-related phenolics during growth, caused by abrasion from the soil. *Agrobacterium* may be attracted to plants by recognition of these compounds. However, significant levels of *vir* induction and the events of T-DNA transfer will only occur if signal molecules are present in sufficient concentrations. As the highest concentrations are found at wound sites, *A. tumefaciens* effectively infects only these sites.

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The *Drosophila* developmental gene, *engrailed*, encodes a sequence-specific DNA binding activity

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Plasmid expression vectors carrying either the entire engrailed coding region or a subfragment including the homoeo box, produce protein fusions having sequence-specific DNA binding activity.

MUTATIONS in *Drosophila* have identified genes that control major steps in development¹⁻³. Some of these mutants, the segmentation mutants, are defective in the processes that subdivide the embryo into the segmented body plan⁴⁻⁷ while others, the homoeotic mutants, improperly specify the developmental fate of particular regions of the fly¹. Garcia-Bellido⁸ suggested that these mutations affect 'selector genes' that act, in those cells in which they are expressed, to select the developmental pathway. It was proposed that they function by controlling 'cytodifferentiation genes'⁸.

A segmentation gene

Each morphologically obvious segment is composed of cells from two distinct lineages termed anterior and posterior compartments^{9,10}. Genetic analysis suggests that the *engrailed* gene product is required to specify cells as members of posterior compartments¹¹⁻¹⁴. As anticipated by the selector gene hypothesis⁸, by 3.5 h of development *engrailed* gene product accumulates in narrow bands corresponding to the primordia of the posterior part of each segment¹⁵⁻¹⁷. Apparently, the

engrailed regulatory activity acts wherever it is expressed to direct cells along a pathway of development suited to cells of posterior compartments⁸. In *engrailed* mutants, the segmental fusions and the failure to specify cells of posterior compartments are thought to result from the absence of this regulator or from alterations in its expression.

Selector genes interact

In addition to *engrailed*, several other *Drosophila* developmental genes are expressed in spatially restricted patterns consistent with their apparent roles in directing particular portions of the developmental programme¹⁵⁻¹⁹. These studies have focused interest on two related issues. How are these genes regulated to achieve the appropriate pattern of expression, and how do they regulate subsequent development? Recent studies suggest that the selector genes interact in a complex regulatory network. Based on phenotypes of double mutant combinations, Struhl²⁰ argued that the *Ubx* gene product represses *Scr* expression in the mesothorax. Molecular studies²¹ have offered further suggestions of interactions. Regulatory interactions among six different

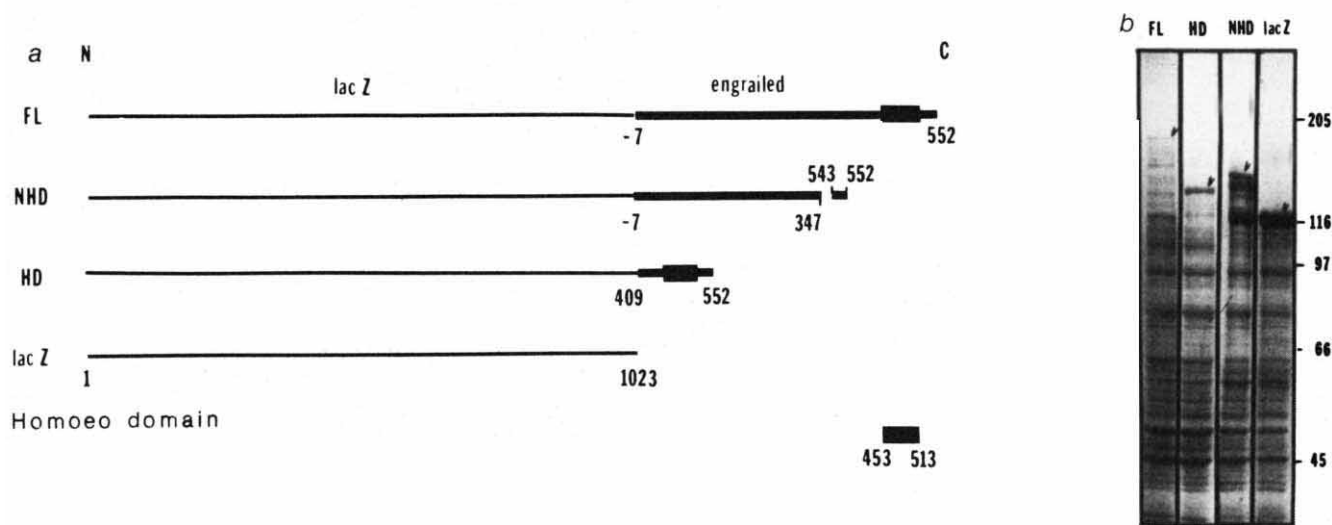


Fig. 1 Construction of *lacZ-engrailed* fusions and expression in *Escherichia coli*. **a**, Gene fusions; **b**, polyacrylamide gel electrophoresis of bacterial extracts; FL, full-length fusion; HD, homoeo domain fusion; NHD, non-homoeo domain fusion; *lacZ*, β -galactosidase.

Methods: **a**, DNA fragments derived from the *engrailed* locus^{17,35,38} were inserted in the polylinker of expression vector plasmids pUR 290, 291 or 292 (ref. 47). These different plasmids allow inserted DNA to be expressed as a C-terminal extension of β -galactosidase. In the full-length fusion protein (FL) the extension includes 7 amino acids that precede the first methionine of the *engrailed* protein as well as the entire *engrailed* protein. In the homoeo domain construct (HD), a *Bam*HI fragment was spliced out of the FL construction, keeping only the last quarter of the *engrailed* coding sequence from amino acid 409 to the end. This fusion protein contains the entire homoeo domain (amino acids 453-513). Amino acids 347-542, containing the homoeo domain, have been deleted in the non-homoeo domain protein (NHD) by removing a *Xho*I fragment from the FL construct. In the absence of insertion the *lacZ* gene is expressed as the complete β -galactosidase. **b**, Polyacrylamide gel electrophoresis of bacterial extracts. The expression of the fusion proteins from chimaeric plasmids is under the control of the *lac* promoter in a *lacI* overproducing strain DG101 (a gift from D. Gelfand, Cetus Corporation). Bacteria in exponential growth were induced with isopropyl β -thiogalactoside at an absorbance of 0.5 (at 600 nm) and collected 2 h later. Cells were resuspended in about 0.005 culture volume of 25% sucrose, 0.2 mM EDTA, 40 mM Tris-HCl pH 7.5 and 1 mM dithiothreitol (DTT). Lysis and protein solubilization were achieved by lysozyme treatment (0.4 mg ml⁻¹ for 1 h at 0°C followed by addition of urea to 4 M and further incubation at 0°C for 1 h). After centrifugation at 20,000 r.p.m. for 1 h the urea was removed from the supernatant by dialysis first against 10 mM Tris-HCl pH 7.5, 25 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM DTT, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, and 0.1 mM benzimidazole containing 2 M urea and subsequently against the same buffer without urea. Glycerol was added to the extracts to a final concentration of 50% and they were stored at -20°C. When analysed by SDS-gel electrophoresis the fusion proteins are seen as abundant high relative molecular mass proteins (arrowheads). The larger fusion protein is present in lower amounts and we detect numerous minor bands that are presumed to result from degradation of the fusion proteins.

homoeotic loci appear to coordinate their spatial patterns of expression (ref. 22 and C. Wedeen and M. Levine, personal communication).

Recent molecular analyses suggest that the segmentation genes interact to control the expression of one another. Immunofluorescent staining revealed that *engrailed* protein appears first in alternate segments and only later in every segment¹⁷. This led to the suggestion that *engrailed* expression is regulated by the products of another class of segmentation genes, the pair-rule genes^{17,23}, which are expressed in alternate segments^{19,24}. Alterations in the pattern of *engrailed* expression in pair-rule mutants have confirmed this prediction (S. DiNardo and P.H.O'F., unpublished observations; K. Howard and P. Ingham, personal communication). Similar analyses of *fushi tarazu* expression suggest that its expression is also influenced by several other segmentation genes (S. Carroll and M. Scott, personal communication).

These observations suggest an extensive network of regulatory interactions among the *Drosophila* developmental genes, and imply that selector genes are themselves targets for the regulators they encode^{17,23}.

The homoeo box

The products of a number of the developmental genes include a conserved protein domain of 60 amino acids, the homoeo domain. Related sequences have been identified in species from human to annelids²⁵⁻³⁰. This remarkable conservation suggests that the homoeo domain has common physical interactions in all these organisms. A portion of this domain is found in two yeast proteins, $\alpha 1$ and $\alpha 2$, that determine the cell fate (mating type) via transcriptional regulation^{31,32}. Because of this homology it has been proposed that the developmental genes of *Drosophila* might function similarly, by interacting with DNA^{27,33}. Further, it has been noted that sequences within the homoeo domains are compatible with a protein structural motif that characterizes bacterial sequence-specific DNA binding proteins^{27,34}.

Here we address three issues. Does a homoeo-domain-containing protein bind to DNA? Is the binding specific? Is the homoeo domain responsible for binding?

Construction of *engrailed* fusion proteins

To study how the *engrailed* protein product might function as a regulator, we constructed bacterial expression vectors that encoded the *engrailed* protein as carboxy-terminal extensions of β -galactosidase. In order to test for possible autonomous functions of the homoeo domain, we constructed three fusion proteins (Fig. 1). The full-length fusion contained the sequence encoding the entire 552 amino acids of the *engrailed* protein. This *engrailed* protein sequence has been derived from an open reading frame in the *engrailed* cDNA sequence³⁵. The pattern of evolutionary sequence conservation of this open reading frame suggests that it encodes protein (J. Kassis, D. K. Wright, and P.H.O'F., unpublished observations). We think that this predicted protein is made *in vivo* because two antisera directed against different domains of this predicted sequence detect expression of these domains in the posterior compartments of segments¹⁷. The 'homoeo domain fusion' includes only the terminal quarter of the protein coding sequence, encompassing the 60-amino-acid homoeo domain and an additional 44 amino acids on the N-terminal side plus 39 amino acids on the C-terminal side. The 'non-homoeo domain fusion' is deleted for a 196-amino-acid region and lacks the homoeo domain (Fig. 1).

Nonspecific DNA binding

We first tested whether the fusion proteins would bind DNA nonspecifically. We mixed the fusion proteins with labelled restriction fragments of DNA and then, using an antibody directed against bacterial β -galactosidase and fixed *Staphylococcus aureus* as an immunoadsorbent, we precipitated the fusion protein along with bound DNA fragments^{31,36}. The bound DNA fragments were then separated by electrophoresis and detected

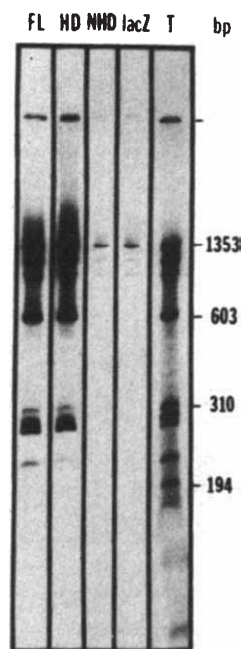


Fig. 2 Nonspecific binding of the fusion proteins to DNA. Bacteriophage Φ X174 DNA was cleaved by *Hae*III and end-labelled using T4 polymerase⁴⁸. The labelled DNA (about 30 ng) was incubated for 30 min at 0°C in 25 μ l of binding buffer (BB) (50 mM NaCl, 20 mM Tris pH 7.6, 0.25 mM EDTA, 1 mM DTT, 10% glycerol) in the presence of a bacterial extract containing the fusion protein (about 10 μ g of total protein extract, see Fig. 1 legend). The complexes formed between DNA and the fusion proteins were then immunoprecipitated by 30 min further incubation at 0°C with an anti- β -galactosidase monoclonal antibody (provided by Tom Mason and Judy Partaledis, courtesy of Mike Hall) preadsorbed on cross-linked *Staphylococcus* (Pansorbin, Calbiochem). The pellet was washed twice in BB, phenol extracted and the DNA ethanol precipitated before polyacrylamide gel electrophoresis and autoradiography. The autoradiogram shows the results of precipitations performed in the presence of extracts containing the full-length fusion (FL), the homoeo domain fusion (HD), the non-homoeo domain fusion (NHD) or β -galactosidase (*lacZ*). Lane T shows the labelled digest before immunoprecipitation, representing 25% of the counts added to the incubation mixture in the immunoprecipitation experiments.

by autoradiography. At low salt concentration (50 mM NaCl) and in the absence of carrier DNA, the full-length fusion protein and the homoeo domain fusion protein bound all of the *Hae*III restriction fragments of bacteriophage Φ X174 DNA, whereas neither β -galactosidase alone, nor the non-homoeo domain fusion protein bound significant amounts of DNA (Fig. 2). Though these observations are consistent with the hypothesis that the homoeo domain would function in DNA binding, the results could potentially be due to simple ionic interactions. Sequence-specific binding would suggest that the fusion protein has a DNA binding domain.

Sequence-specific DNA binding

Since we had no knowledge of what the target sequences for binding of *engrailed* protein might be, we sought a generalized approach to detect sequence specificity. Sequence-specific DNA binding proteins recognize degenerate versions of a consensus binding site. Sufficiently complex DNA should contain, by chance, sequences recognized by the protein. For example, Ross and Landy³⁷ identified the sequence of several binding sites for λ integrase (Int protein) in pBR322 DNA.

To pursue this approach, we digested λ -phage DNA to produce more than 100 fragments and labelled their 3' ends. This DNA was used in the assay described above, except that we added increasing amounts of salt or carrier DNA so that only fragments that bound to the fusion proteins with higher affinities would appear in the precipitate. Figure 3 shows that at low concentrations of carrier DNA all the λ DNA fragments are bound nonselectively (lanes 2, 5), but as the stringency of the

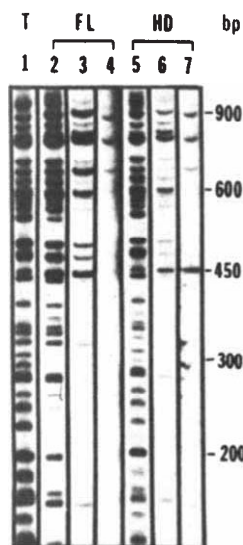


Fig. 3 Sequence-specific DNA interaction of the fusion protein with bacteriophage λ DNA fragments. Bacteriophage λ DNA was restricted with *Sau*3A and labelled using T4 polymerase⁴⁸. Binding assays were performed as described in Fig. 2 legend with the addition of carrier DNA as indicated. Fragments were separated on a 5% polyacrylamide gel. Lane 1 shows the total digest. The amount of DNA loaded in lane 1 is one-quarter of the input amount used in the binding assays (10 ng; $0.4 \mu\text{g ml}^{-1}$). Lanes 2, 3 and 4 show immunoprecipitates obtained following addition of the full-length fusion extract in the presence of 0, 4 and $40 \mu\text{g ml}^{-1}$ of carrier DNA (fragmented calf thymus DNA), respectively. Lanes 5, 6 and 7 show the results of a similar experiment using the homoeo domain fusion extract. Lanes 4 and 7 were exposed four times longer because of the reduced recovery of bands at high levels of carrier.

binding conditions is increased the binding becomes selective. For example, at high concentration of carrier DNA (Fig. 3, lanes 4, 7), only 4 fragments out of 115 DNA fragments are retained. These experiments demonstrate the specificity of *engrailed* fusion protein binding to DNA.

Whether the homoeo domain fusion or the full-length fusion is used in binding assays, the same fragments are recovered in immunoprecipitates at comparable efficiencies (Fig. 3). Thus, the two fusions bind with the same specificity and generally exhibit similar relative affinities for these fragments. The 143 amino acids of the *engrailed* sequence present in the homoeo domain fusion must include a domain competent in specific binding. At least under the conditions of our assay, the additional 409 amino acids of the full-length fusion protein make little or no contribution to the specificity of binding.

To estimate the minimal binding constant of the binding interaction, we assume that all of the fusion protein is active^{31,36}. When our binding assay contains less than 10^{-8} M fusion protein and less than 10^{-11} M DNA fragments, recovery of specific DNA fragments in the immunoprecipitate exceeds 50% (for example Fig. 4A, *engrailed* fragments f and k). Accordingly, the binding constant must exceed $5 \times 10^7 \text{ mol}^{-1}$ (at 170 mM NaCl). Furthermore, preliminary evidence indicating that only a fraction of the fusion protein is active (J.T., unpublished observations) suggests that the binding constant must be higher, and may well be comparable to the binding constants of other sequence-specific DNA binding proteins³⁴.

The binding behaviour of the fusion protein described here may not accurately reproduce the behaviour and specificities of the natural *engrailed* protein. The binding specificity might be influenced by interactions that the fusion protein cannot reproduce or by modifications that would be missing in a protein produced in *Escherichia coli*. Furthermore, our simple *in vitro* binding assay may lack accessory factors that influence *in vivo* binding of the *engrailed* protein to DNA. Nonetheless, because other work using fusion proteins or proteolytic fragments^{31,34} suggests that DNA binding domains can function relatively autonomously, we believe that the results reported here are likely to reflect at least a subset of the activities of the normal *engrailed* protein.

Specific binding to *Drosophila* DNA

If *engrailed* and other selector genes act as pleiotropic regulators of transcription, we might expect their protein products to interact with DNA near the promoters of a number of target genes. Can we identify any plausible candidates for target genes? There is considerable evidence that selector genes regulate each other's expression (summarized above). Thus, we envision that the developmental genes will include regulatory sites that are targets for interaction with the products of other developmental genes. Because of the high degree of relatedness of the developmental genes, the various target sites might also be homologous. Because it is a member of this group of related proteins, perhaps the *engrailed* fusion protein will exhibit site-specific interaction with all or a subset of the related regulatory sites.

Following this line of logic, we decided to look for *engrailed* fusion protein binding adjacent to cloned selector genes. Because a detailed analysis would require DNA sequence information, we chose to examine the *engrailed* locus itself, for which we have 1.2 kilobases (kb) of upstream sequence (unpublished data), and the *fushi tarazu* locus that had been sequenced by Laughon and Scott²⁷.

We looked for *engrailed* fusion protein binding to a 4.9-kb *Eco*RI fragment that includes 2.6 kb of *engrailed* coding sequence and 2.3 kb of upstream sequences³⁸ and to a 3.2-kb fragment that includes the *fushi tarazu* coding sequence and flanking sequences⁴⁹. Figure 4A shows that both the cloned *engrailed* sequences and cloned *fushi tarazu* sequences contain fragments that bind to the *engrailed* fusion protein under stringent assay conditions. In fact, a number of binding fragments are detected (Fig. 4A, C). The positions of binding fragments are indicated in Fig. 4B.

We purified the subfragments indicated by the hatched lines in Fig. 4B and used these to map more precisely the binding interactions upstream of the *engrailed* and *fushi tarazu* coding regions. Secondary digests of these fragments were tested for interactions with the *engrailed* fusion protein (Fig. 5). These analyses localized three binding sites within the 900-base-pair (bp) region 5' to the *engrailed* cDNA. The higher resolution and sensitivity of these experiments showed that the binding fragment k (Fig. 4A, B) actually contained two binding sites (sites a and b in Fig. 5) and that fragment d, though not detected as a binding fragment in experiments using the whole plasmid, contains a weak binding site (site c in Fig. 5). The analysis of the *fushi tarazu* subfragment did not reveal any new binding sites but did contribute to more accurate localization of the upstream site (Fig. 5). At present the accuracy of localization of the sites does not allow us to identify a consensus binding site unambiguously.

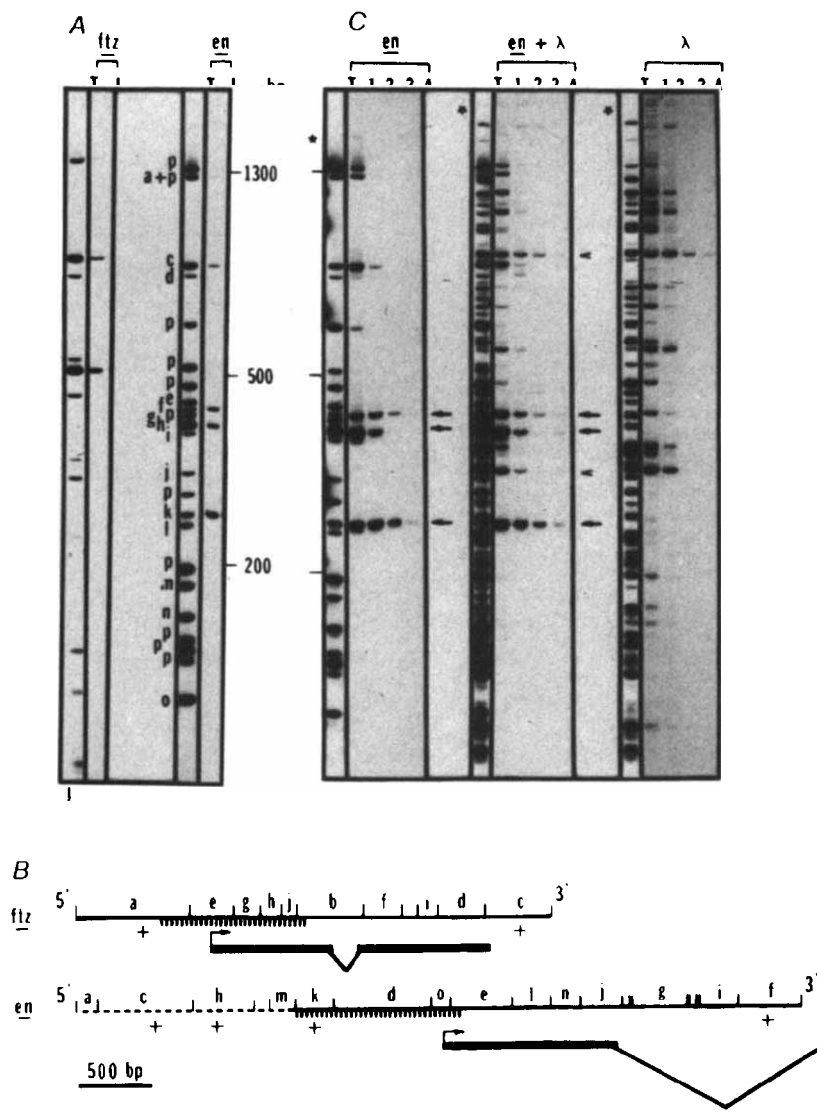
Binding sites

Without a functional assay we cannot directly assess the importance of the binding sites detected in cloned *Drosophila* sequences. However, we can test whether affinities, frequency of occurrence, clustering and location of binding sites differ from fortuitous sites.

If the frequency of fortuitous sites were extremely low (less than 1 per 1,000 kb), the presence of a cluster of binding sites within a few kilobases of *engrailed* DNA would be highly significant. This is not the case. Although the frequency of binding sites on a 4.9-kb fragment of *engrailed* DNA is higher than the density of λ DNA, the difference was not very large—about 10-fold (Fig. 4C).

Fortuitous binding sites should have a wide range of affinities depending on their similarity to an optimal site. Higher-affinity fortuitous sites should be less frequent (chance might produce an optimal binding site but should do so less frequently than imprecise approximations of this sequence). We tested the relative affinity of the *engrailed* fusion protein for various binding sites. We used conditions where a number of labelled restriction fragments are bound selectively. Addition of cold competitor DNA displaced bacteriophage λ DNA fragments with different

Fig. 4 Sequence-specific interaction of the homoeo domain fusion protein with restriction fragments of cloned *fushi tarazu* and *engrailed* sequences. The *fushi tarazu* clone (p6-3, derived from clone pDm439, a gift from Matt Scott)⁴⁰ contains 900 bp of upstream sequence, the entire coding sequence and 800 bp of downstream sequence. The *engrailed* clone (p615) contains 2.3 kb of upstream sequence, the complete first exon and most of the first intron^{35,38} (panel B). Restriction fragments of p6-3, p615 or λ DNA were end-labelled and tested for fusion protein binding as described in the legend for Fig. 2, except that a higher salt concentration (170 mM NaCl) was used to diminish nonspecific interactions. Fragments were separated on a 5% acrylamide gel. In the example shown in A, the *fushi tarazu* plasmid (ftz) DNA was digested with *RsaI* and the *engrailed* plasmid (*en*) was digested with *HinfI* and *Clal*. Lanes marked T show the total fragment pattern and lanes marked I show the fragments recovered in the immunoprecipitate. For both plasmids the amount of DNA loaded in T is one-quarter of the amount subjected to immunoprecipitation and displayed in I. Bands derived totally from plasmid sequences are identified with a 'p'. Bands derived at least in part from insert sequences are designated as a to j (ftz) or a to o (en) and their positions within the cloned sequence are indicated in B. Similar experiments using *Afl*III, *Hae*III, *Bst*NI, *Dde*I or *Hpa*II to digest these plasmids gave comparable results (not shown). B shows a map of the inserts in p6-3 (ftz) and p615 (en). The orientation of the coding region within the insert is indicated. Except for the dashed portion of the *engrailed* insert, the sequence is known (refs 27, 35, and J. Kassis, D. K. Wright and C.D., unpublished). The positions of restriction enzyme sites (*RsaI* sites in ftz and *HinfI* and *Clal* sites in en) are indicated and all fragments detectable in the separation shown in panel A are lettered. Those fragments detected as bound by the homoeo domain fusion protein are indicated as +. Because they were not detected, a few small restriction fragments (unlettered) could not be scored in the binding experiment (A) but analysis of these DNAs cleaved with other restriction enzymes failed to detect additional binding sites. Exons of the transcribed region are indicated with a bold line and introns with sloping lines. The hatched region indicates the position of a subfragment that was purified and used for additional binding studies (Fig. 5). Note that the region responsible for binding of ftz fragment a, was further localized by analysis of *Afl*III, *Hae*III, *Bst*NI, *Dde*I, *Hpa*II and *HinfI* digests that defined only one site; this site corresponds to that mapped in more detail by analysis of the purified subfragment (Fig. 5). C Compares the efficiency of homoeo domain fusion protein binding to *engrailed* or λ sites. The binding assays were performed as described in A except that increasing amounts of unlabelled DNA (*HinfI*-restricted p615) was added as competitor. Lanes T represent 10% of the counts of the total digest submitted to immunoprecipitation in lanes 1, 2, 3 and 4. In lane 1, no unlabelled DNA was added while in lanes 2, 3 and 4, 30 ng, 250 ng and 4 μ g, respectively, were added. en lanes: plasmid p615 was restricted with *Clal* and *HinfI*, labelled and submitted to immunoprecipitation. Lanes 1, 2, 3 and 4 represent immunoprecipitated DNA from ~4 ng of labelled DNA (~1 fmol). Obviously the four bands retained in A behave differently when unlabelled competitor DNA is added. Three bands (arrows) corresponding to fragments f, k and h in A, are detected in the immunoprecipitate in the presence of 1,000-fold excess of unlabelled DNA. λ lanes: λ DNA was restricted with *Clal* and *HinfI*, labelled and processed under the same conditions as for en DNA. An equimolar amount of DNA (1 fmol) was used in this experiment (20 ng). Two bands were retained at the highest stringency (arrowheads). en + λ lanes: T is an equimolar mixture of en and λ DNAs restricted with *Clal* and *HinfI*. The bands that are retained are the same as those seen in the experiments testing en and λ DNAs individually. Five bands are retained in the presence of the highest concentration of competitor (arrows and arrowheads).



efficiencies (Fig. 4C). Thus, as expected, the binding sites in λ DNA have a range of affinities and there are few high-affinity sites. Assuming that the binding sites in λ DNA occur by chance, the specific binding of 14 restriction fragments at intermediate stringencies suggests that the sequence recognized by the *engrailed* fusion protein is relatively short (about 6 bp) or substantially degenerate.

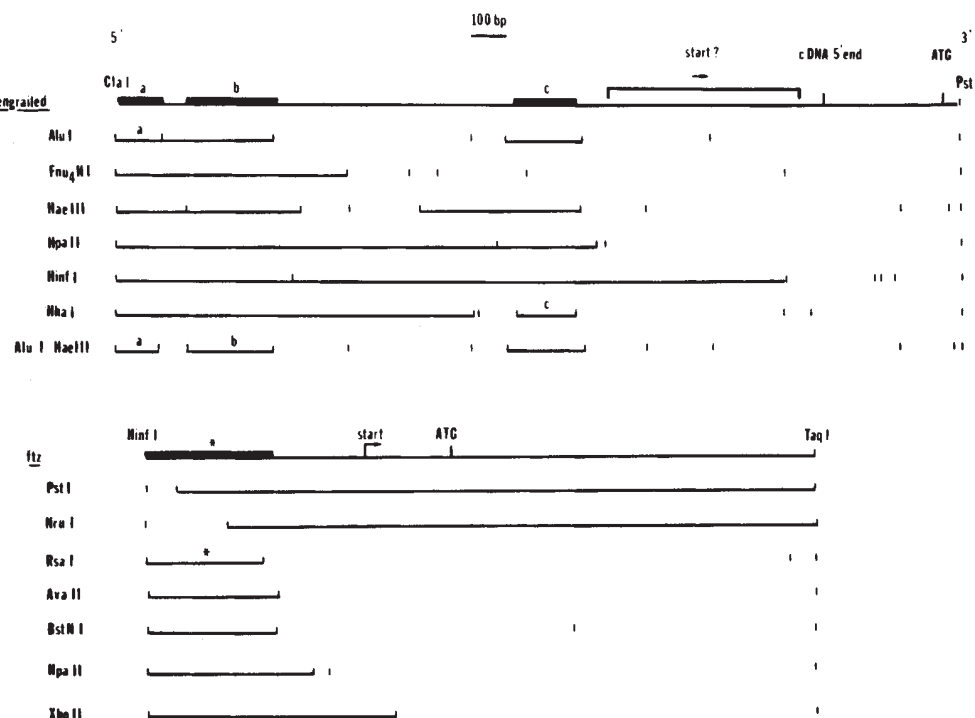
For some sequence-specific DNA binding proteins (such as *lac* repressor), fortuitous occurrence of high-affinity binding sites is extremely unlikely. For these a site of functional interaction (the *lac* operator³⁹) has a distinctively high affinity. For other sequence-specific interactions (for example λ integrase^{37,40}) the affinities of fortuitous and functional sites overlap. Depending on the characteristics of the *engrailed* fusion protein, functionally relevant sites might have distinctively high affinities. We therefore examined the relative binding affinity of *engrailed* and bacteriophage λ DNA fragments. We observe differing affinities for the *engrailed* fusion protein interaction with various sites on *engrailed* DNA (Fig. 4C). Using our present assay, the ranges of affinities seen for λ and *engrailed* sites overlap (Fig. 4C). Three *engrailed* fragments bind with

particularly high affinity (arrows) compared with two λ fragments (arrowheads).

The binding data provide no support for suggestions that the binding sites in *Drosophila* DNA are functional. It should, however, be made clear that the opposing conclusion also cannot be reached from these data; that is, the binding sites in *engrailed* DNA cannot be dismissed as nonfunctional because they have properties similar to fortuitous binding sites. Thus, the issue of function remains open.

Although the functional importance of the binding sites still requires experimental test, we propose that the binding sites we have detected in *Drosophila* DNA function *in vivo* as targets for interaction with either the *engrailed* protein or closely related gene products (that is, other selector genes). We make this suggestion on the basis of the location, clustering and conservation of the sites. The positions of binding sites in relation to the *fushi tarazu* and the *engrailed* coding regions are reminiscent of the positions of enhancer elements in other systems⁴¹⁻⁴³. The clustering of binding sites is unlikely to be coincidental. Such clustering of binding sites for regulatory proteins is fairly common (for example, refs 44, 45). Finally, if functional, the binding

Fig. 5 Localization of binding sites in the 5' regions of *engrailed* and *fushi tarazu*. In digests of whole plasmid DNA we identified fragments that were bound by the homoeo domain fusion protein (Fig. 4). To localize more precisely the binding sites immediately upstream of the *engrailed* and *fushi tarazu* coding regions, we purified a 1,180-bp *Cla*I-*Pst*I fragment from the *engrailed* clone and a 939-bp *Hinf*I-*Taq*I fragment from the *fushi tarazu* clone (Fig. 4B). These purified fragments were digested with additional enzymes as indicated and the binding of subfragments assayed as before (Fig. 4). In each of the restriction digests shown here the binding fragments are indicated as solid lines. Although the overlap of binding sites: a *Cla*I-*Alu*I fragment (a, the binding regions, this could be misleading if the binding sites were complex. Therefore, in the summary diagrams above the restriction patterns, we have indicated the precision of the localization of the binding sites based on the minimal fragment showing binding (bold line). For *engrailed*, three fragments were identified as containing binding sites: a, *Cla*I-*Alu*I fragment (a, 67 bp), a *Hae*III-*Alu*I fragment (b, 100 bp) and a *Hha*I fragment (c, 88 bp). For *ftz* a single site was localized to a 165-bp *Hinf*I-*Rsa*I fragment(*).



sites will be conserved in evolution. In the absence of a functional test, we believe that the best way to distinguish fortuitous and functional binding sites is to see whether protein binding occurs at analogous positions in distantly related genomes. We have cloned the *engrailed* gene of a distantly related *Drosophila* species, *D. virilis*⁴⁶. A preliminary analysis indicates that the fusion protein binds to fragments upstream of the *D. virilis engrailed* gene (D. Wright, unpublished data).

Binding specificity

Together with previous arguments²⁷, our results predict that the homoeo domain imparts a sequence-specific DNA binding activity to the protein. Accordingly, other homoeo domain-containing proteins should also bind DNA in a sequence-specific manner and such proteins having closely homologous homoeo domains should have similar sequence specificity. We presently recognize two classes of homoeo domain sequences. Class I is comprised of seven genes which have highly homologous homoeo domains and are located in two clusters of developmental genes (the bithorax complex and the *Antennapedia* complex)^{22,25}. Class II is comprised of the *engrailed* homoeo domain and the highly homologous homoeo domain of the *engrailed* related gene³⁵. The homoeo domains of different classes have lower homology (Fig. 6). As noted previously, the regions of sequence identity suggest that class I homoeo domains might specify binding to the same sequence²⁷. The differences between class I and class II homoeo domains might include an alteration of the sequence specificity.

Evolution of proteins

Duplication and divergence of a primordial gene encoding a DNA binding protein might lead to a family of interacting regulators. If the primordial protein included sequences for dimerization and for DNA binding, newly duplicated coding regions would have common binding specificities and could form heterotypic dimers. The interactions between products of duplicated genes would persist if the dimerization function and DNA binding function were conserved. We suggest that continued duplication and divergence can result in a family of DNA binding proteins that interact physically by forming heterotypic associations and interact functionally by competition for binding

to related DNA binding sites. Such interactions would link the various genes in a regulatory network. The evolution of the members of the family would be coupled because of the importance of maintaining the interactions among the members of this regulatory network. Since coordinate change of many genes is an extraordinarily unlikely event, such a network of interaction

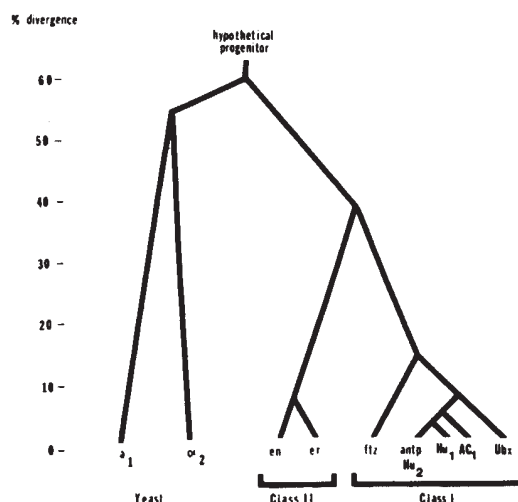


Fig. 6 Family tree of relatedness of homoeo domains. Pairwise comparisons of the protein sequences of the homoeo domains encoded by *Drosophila* genes (*Antp*, *Ubx*, *ftz*, *en* and *er*), yeast genes ($\alpha 1$ and $\alpha 2$) and sequences isolated by homology from humans (Hu_1 and Hu_2) and frogs (AC_1) were used to score divergence. The comparison was confined to residues 29-58 because this region is well conserved among all of these sequences. To produce homology scores we gave one point for amino-acid identity and half a point for similarity. The data are assembled into a family tree by indicating the divergence of each sequence that approximates all the pairwise comparisons. The sequence data are taken from Levine *et al.*²⁸ (Hu_1 and Hu_2), Scott and Weiner²⁶ (*Ubx* and *ftz*), McGinnis *et al.*²⁵ (*Antp*), Poole *et al.*³⁵ (*en* and *er*), Carrasco *et al.*³⁰ (AC_1), and Tatchell *et al.*³² ($\alpha 2$ and $\alpha 1$). Note that the discrepancies in the published sequence^{25,26} for *Ubx* are significant in comparison to the divergence among class I homoeo domains.

may contribute to the extraordinary conservation of homoeo domain sequences. It should be possible to test the predictions of this rationale using approaches similar to those used here to show that the *engrailed* gene encodes a sequence-specific DNA binding activity.

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Primary structure and expression of a functional human glucocorticoid receptor cDNA

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Identification of complementary DNAs encoding the human glucocorticoid receptor predicts two protein forms, of 777 (α) and 742 (β) amino acids, which differ at their carboxy termini. The proteins contain a cysteine/lysine/arginine-rich region which may define the DNA-binding domain. Pure radiolabelled glucocorticoid receptor, synthesized in vitro, is immuno-reactive and possesses intrinsic steroid-binding activity characteristic of the native glucocorticoid receptor.

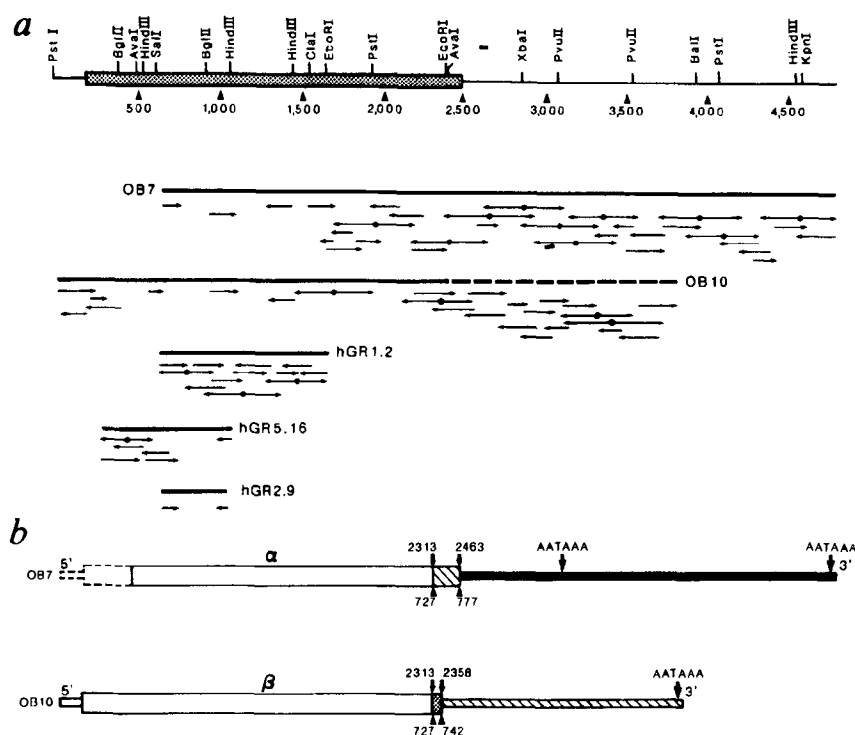
TRANSCRIPTIONAL regulation of development and homeostasis is controlled in complex eukaryotes by a wide variety of regulatory substances, including steroid hormones. The latter exert potent effects on development and differentiation in phylogenetically diverse organisms and their actions are mediated as a consequence of their interactions with specific, high-affinity binding proteins referred to as receptors¹⁻⁶. Many of the primary effects of steroid hormones involve increased transcription of a subset of genes in specific cell types^{7,8}. The structural characterization of the steroid receptor protein and the definition of the molecular actions of the steroid hormone/receptor complex would help to define the biochemical events which modulate gene transcription. A series of receptor proteins, each specific for one of several known steroid hormones, are distributed in

a tissue-specific fashion, although many cell types simultaneously express receptors for several steroid hormones^{9,10}. It is postulated that steroids enter cells by facilitated diffusion and bind to the specific receptor protein, initiating an allosteric alteration of the complex. As a result of this transformation, the steroid hormone/receptor complex appears capable of binding high-affinity sites on chromatin and modulating transcription of specific genes by a mechanistically unknown process^{11,12}.

The glucocorticoid receptor is widely distributed and expressed in many cultured cell lines, and the control of gene expression by glucocorticoids, therefore, has been widely studied as a model for transcriptional regulation. A number of glucocorticoid-responsive transcription units, including mouse mammary tumour virus (MMTV)^{13,14}, mouse and human metal-

Fig. 1 Human glucocorticoid receptor cDNA sequencing strategy and schematic representation of cDNA clones. *a*, The composite cDNA for the α glucocorticoid receptor is represented at the top, with noncoding (lines) and coding (stippled portion) sequences indicated. Common 6-nucleotide restriction enzyme sites are shown. Overlapping cDNA inserts used to determine the sequence are shown: arrows beneath the regions sequenced show the direction and extent of sequencing. The dashed line at the 3' end of OB10 indicates divergent sequence. Numbers refer to nucleotide positions in OB10 relative to the 5'-most transcribed sequence. *b*, cDNAs encoding the α and β forms of the receptor (OB7 and OB10, respectively). The 5' end of OB7 (broken lines) is contributed by the OB10 clone. Protein-coding information is represented by wide bars; untranslated sequences are indicated by thin bars. Nucleotides and amino acids are numbered above and below the coding sequence, respectively. Common DNA sequences extend to nucleotide 2,313 (amino-acid residue 727), at which point the α - and β -receptor forms diverge, with the α cDNAs (OB12, OB7) continuing in an open reading frame for 150 nucleotides (50 amino acids) and the β cDNA (OB10) continuing for 45 nucleotides (15 amino acids; see Fig. 3). Hexanucleotide signals (AATAAA) just upstream of the poly(A) in the clones are indicated, with the first hexanucleotide in OB7 serving as poly(A) in OB12.

Methods. The inserts hGR1.2, hGR2.9 and hGR5.16 were isolated from a λ gt11 IM-9 lymphoid cell cDNA library as described previously⁴². Two clones were isolated from cDNA libraries constructed by H. Okayama in pcD (ref. 44) using poly(A)⁺ mRNA from GM637 human fibroblasts (OB7) and primary human fibroblasts (OB10). Screening was performed with the hGR1.2 cDNA, radiolabelled by nick-translation with ³²P-dCTP. Sequences were determined by the chemical cleavage method of Maxam and Gilbert⁴⁵.



lothionein^{15,16}, rat α_2 -globulin¹⁷ and rat and human growth hormone¹⁸⁻²⁰ genes have been identified. DNA sequences mediating transcriptional stimulation of several of these genes have been localized. For MMTV, these sequences are discrete genomic regions upstream of the transcriptional start site which appear to exert their actions independently of orientation and position^{21,22}. The steroid/receptor complex appears to bind to these regulatory sequences and purified receptor has been used to define the specific binding sites²³⁻²⁶. Based on the footprinting analyses of several responsive genes, a consensus DNA binding sequence sharing the core sequence 5' TGT/CTCT 3' has been proposed²⁷.

The ability of the glucocorticoid-responsive element (GRE) to alter its position and orientation yet still maintain promoter inducibility suggests that it resembles the class of *cis*-acting regulatory sequences termed enhancers²¹. First discovered in viruses and subsequently in cellular genes, these sequences are necessary for efficient transcription *in vivo*²⁸⁻³¹. It has been suggested that enhancers are recognized by *trans*-acting factors that mediate regulatory effects by tissue-specific transcriptional control. Although the enhancer factors have not been well characterized, the glucocorticoid receptor may serve as a paradigm for these putative gene activator proteins.

The availability of radiolabelled high-affinity glucocorticoid analogues such as dexamethasone and triamcinolone acetonide has led to the development of purification strategies resulting in the isolation of nearly pure rat and human receptors^{32,33}. Although the receptor migrates as a dimer in sucrose gradients, analysis on denaturing SDS-polyacrylamide gels detects a single polypeptide of relative molecular mass (M_r) ~94,000 (94K)^{34,35}. The native polypeptide contains intrinsic specificity for steroid binding and DNA sequence recognition. By using as probes monoclonal and polyclonal antibodies raised against the purified rat and human receptors³⁶⁻³⁸, it has been possible to identify a major immunogenic region in the receptor residing on a portion of the molecule that is distinct from the steroid- and DNA-binding regions³⁹⁻⁴¹. To gain further information about the structure of this molecule and to begin an analysis of the

molecular mechanisms by which it regulates gene transcription, we set out to clone receptor cDNA sequences. By using receptor-specific antibodies as probes, we and others have isolated clones containing human or rat glucocorticoid receptor cDNA inserts^{42,43}.

Here we report the complete amino-acid sequence of the human glucocorticoid receptor (hGR), deduced from human lymphoid and fibroblast cDNA clones. The sequence reveals various structural features of the receptor, including the major immunogenic domain and a cysteine/arginine/lysine-rich region which may constitute a portion of the DNA-binding domain. We describe the use of the SP6 transcription vector system to generate analytical amounts of full-length protein, and demonstrate that the cell-free translated protein is both immunoreactive and possesses steroid-binding properties characteristic of the native glucocorticoid receptor. An accompanying paper describes the homology of the hGR sequence to that of the oncogene *v-erbA*⁶⁴.

Glucocorticoid receptor cDNA

A library of cDNA clones was constructed in the phage expression vector λ gt11 using poly(A)⁺ RNA from the human lymphoid cell line IM-9 as template, as described previously⁴². This library was initially screened with a rabbit polyclonal antiserum to the purified glucocorticoid receptor, resulting in the isolation of several immunopositive candidate clones from $\sim 2.5 \times 10^5$ plaques. The β -galactosidase fusion proteins generated from these clones were used to affinity-purify receptor epitope-specific antibody, which was subsequently recovered and identified by binding to protein blots of cellular extracts. Three clones containing inserts expressing antigenic determinants of the human glucocorticoid receptor were isolated. The inserts of these clones, although of different sizes, cross-hybridized, indicating that they contained a common sequence which presumably delimits the major immunogenic domain of the receptor. Together, these clones spanned 1.4 kilobase pairs (kbp) but were clearly not long enough to code for the entire

1	TTTTAGAAAAAAATATATTTCCTCTGCTCCTTCGTCGCTTCAACAGTAAAGTTGTTTATCTCGGCTCGCGCGGAAGCTCGGACGGTGGCGGGGAGCGGCTCCTCTGCCAGGT	120		
2	MetAspSerLysGluSerLeuThrProGlyArgGluGluAsnProSerValLeuAlaGlnGluArgGlyAspValMetAspPheThrLysThrLeuArgGlyGly	120		
3	TTGATATTCACTGATGACTCCAAGAACTTAATCTCTGCTAGAGAAAGAAAACCCAGCAGTGTCTGCTCAGGAGAGGGAGATGTGATGGACTTCTATAAAACCCCTAAGAGGAGGA	240		
4	40	50	60	70
5	AlaThrValLysValSerAlaSerSerProSerLeuAlaValAlaSerGlnSerAspSerLysGlnArgArgLeuLeuValAspPheProLysGlySerValSerAsnAlaGlnGlnPro	360		
6	GCTACTGTGAAGGTTTCTGCGTCTTCAACCCTCACTGGCTGCTCTTCAATCAGACTCCAAGCAGCGAAGACTTTGGTTGATTTCCTCAAAGGCTCAGTAAGCAATGCGCAGCAGCA	360		
7	80	90	100	110
8	AspLeuSerLysAlaValSerLeuSerMetGlyLeuTyrMetGlyGluThrGluThrLysValMetGlyAsnAspLeuGlyPheProGlnGlnGlyGlnIleSerLeuSerSerGlyGlu	480		
9	GATCTGTCCAAGCAGTTTCACTCTCAATGGGAGTGTATGGGAGAGACAGAAACAAAGTGATGGGAATGACCTGGGATCCACAGCAGGGCCAAATCAGCTTCTCTGGGGGAA	480		
10	120	130	140	150
11	ThrAspLeuLeuLeuGluGluSerIleAlaAsnLeuAsnArgSerThrSerValProGluAsnProLysSerSerAlaSerThrAlaValSerAlaAlaProThrGluLysGluPhe	600		
12	ACAGACTTAAAGCTTTGGGAAGAAGCATTGCAACCTCAATAGGTGACAGCTTCCAGAGAACCCCAAGATTCCAGACTCCACTCTGCTGCTGCCCAACAGAGAGGAGTTT	600		
13	160	170	180	190
14	ProLysThrHisSerAspValSerSerGluGlnGlnHisLeuLysGlyGlnThrGlyThrAsnGlyGlyAsnValLysLeuTyrThrThrAspGlnSerThrPheAspIleLeuGlnAsp	720		
15	CAAAAACTCACTCTGATGTATCTTCAAGAACCAACATTTGAGGGCCAGACTGGCACCACCGGTGGCAATGTGAATGTATACCAACAGACCAAAGCAGCTTTTGACATTTTGAGGAT	720		
16	200	210	220	230
17	LeuGluPheSerSerGlySerProGlyLysGluThrAsnGluSerProTrpArgSerAspLeuLeuIleAspGluAsnCysLeuSerProLeuAlaGlyGluAspAspSerPheLeu	840		
18	TTGGAGTTTTCTCTGGGTCCCCAGGTAAAGAGACGAATGAGAGTCTCTGGAGATCAGACCTGTGATAGATGAAAACTGTTTCTTCTCTCTGGGGGAGAGACGATTTCATCTCT	840		
19	240	250	260	270
20	LeuGluGlyAsnSerAsnGluAspCysLysProLeuIleLeuProAspThrLysProLysIleLysAspAsnGlyAspLeuValLeuSerSerProSerAsnValThrLeuProGlnVal	960		
21	TTGGGAAGGAACTCGAATGAGGACTCGAAGCCTCTCATTTTACCGGACACTAAACCCAAATTAAGGATAAATGGAGATCTGGTTTTGTCAGCCCCAGTAATGTAACACTGCCCAAGTG	960		
22	280	290	300	310
23	LysThrGluLysGluAspPheIleGluLeuCysThrProGlyValIleLysGlnGluLysLeuGlyThrValTyrCysGlnAlaSerPheProGlyAlaAsnIleIleGlyAsnLysMet	1080		
24	AAAAACAGAAAAGAGATTTCATCGAAGCTCTGACCCCTGGGTAAATTAAGCAGAGAACTGGGCAGACTTTACTGTCAGGCAAGCTTCTCTGGAGCAATATATATGGTAATAAATG	1080		
25	320	330	340	350
26	SerAlaIleSerValHisGlyValSerThrSerGlyGlyGlnMetTyrHisTyrAspMetAsnThrAlaSerLeuSerGlnGlnGlnAspGlnLysProIlePheAsnValIleProPro	1200		
27	TCGCGCAATTTCTGTTTCACTGGTGTGATACCTCTGGAGGACAGATGTACCACTATGACATAGACTCCCTTCTCAACAGCAGGATCAGACAGCTATTTTAAATGTCATTCACCA	1200		
28	360	370	380	390
29	IleProValGlySerGluAsnTrpAsnArgCysGlnGlySerGlyAspAspAsnLeuThrSerLeuGlyThrLeuAsnPheProGlyArgThrValPheSerAsnGlyTyrSerSerPro	1320		
30	ATTCCCGTTGGTTTCGAAAAATGGAAATAGGTGCAAGGATCTGGAGATGACAACTTGACTCTCTCTGGGACTCTGAACCTCCCTGGTCTGACAGCTTTTCTTAATGGCTATTCAGCCCC	1320		
31	400	410	420	430
32	SerMetArgProAspValSerSerProProSerSerSerSerThrAlaThrThrGlyProProProLysLeuValCysSerAspGluAlaSerGlyCysHisTyrGlyValLeu	1440		
33	AGCATGAGACCAAGATGTAGCTCTCTCCATCCAGCTCTCAACAGCAACAACAGGACCCTCCCAACTCTGCTGGTGTGCTCTGATGAGCTTCAGGATGTATTATGAGTCTTTA	1440		
34	440	450	460	470
35	ThrCysGlySerCysLysValPhePheLysArgAlaValGluGlyGlnHisAsnTyrLeuCysAlaGlyArgAsnAspCysIleIleAspLysIleArgArgLysAsnCysProAlaCys	1560		
36	ACTGTGGAGAGCTGAAAGTTTTCTTCAAAGAGCAGTGGAAAGGACAGCACAAATTACCTATGTGTCTGGAAGGAATGATTGCATCATCGATAAAATTCGAAAGAAAACTGCCAGCATGC	1560		
37	480	490	500	510
38	ArgTyrArgLysCysLeuGlnAlaGlyMetAsnLeuGluAlaArgLysThrLysLysLysIleLysGlyIleGlnGlnAlaThrThrGlyValSerGlnGluThrSerGluAsnProGly	1680		
39	CGCTATCGAAAAATGCTTCAGGCTGGAATGAACCTGGAGCTGCAAAAACAAGAAAAAATAAAGGAATTTCAGCAGGCCACTACAGAGCTCTCAAGAAACCTCTGAAAACTCTGGT	1680		
40	520	530	540	550
41	AsnLysThrIleValProAlaThrLeuProGlnLeuThrProThrLeuValSerLeuLeuGluValIleGluProGluValLeuTyrAlaGlyTyrAspSerSerValProAspSerThr	1800		
42	AACAAAAACAATATTTCTGCAAGCTTACCAACAACCTACCCCTACCTGGGTGTCACGTCTGGAGGTTATTGAAACCTGAGTGTATATGCGAGGATATGATAGCTCTGTTCCAGACTCAACT	1800		
43	560	570	580	590
44	TrpArgIleMetThrThrLeuAsnMetLeuGlyGlyArgGlnValIleAlaValLysTrpAlaLysAlaIleProGlyPheArgAsnLeuHisLeuAspAspGlnMetThrLeuLeu	1920		
45	TGGAGGATCATGTACTACGCTCAACATGTTAGGAGGGCGGCAAGTGATGTGCAGCAGTGAATGGGCAAGGCAATACAGGTTTCAGGAACCTACACCTGAGTACCAATGACCCCTACTG	1920		
46	600	610	620	630
47	GlnTyrSerTrpMetPheLeuMetAlaPheAlaLeuGlyTrpArgSerTyrArgGlnSerSerAlaAsnLeuLeuCysPheAlaProAspLeuIleIleAsnGluGlnArgMetThrLeu	2040		
48	CAGTACTCTCGGTATGTTTCTTATGGCATTGTCTCTGGGGTGGAGATCATATAGACAAATCAGTGCAAACTCTGCTGTTTTGCTCCTGATCTGATTATTAATGAGCAGAGAAATGACTCTA	2040		
49	640	650	660	670
50	ProCysMetTyrAspGlnCysLysHisMetLeuTyrValSerSerGluLeuHisArgLeuGlnValSerTyrGluGluTyrLeuCysMetLysThrLeuLeuLeuSerSerValPro	2160		
51	CCCTGCACTGACGACCAATGTAAACACATGCTGTATGTTTCTCTGAGTTACACAGGCTCTCAGGTATCTTATGAGAGTATCTCTGTATGAAAACTTACTGCTTCTCTCTCAGTTCTCT	2160		
52	680	690	700	710

Fig. 2 cDNA and predicted protein sequence of human glucocorticoid receptor. The complete α coding sequence and OB7 3'-untranslated region are shown, with the deduced amino acids given above the long open reading frame. An upstream in-frame stop codon at nucleotides 121-123 and putative additional polyadenylation signals in OB7 are underlined.

receptor, which was estimated to require ~2,500 nucleotides to encode a polypeptide of M_r 94K.

To isolate additional cDNA clones we again screened the original library and also examined a second library (given by H. Okayama) prepared with poly(A)⁺ RNA from human fibro-

lasts in the vector described by Okayama and Berg⁴⁴. Using one of the immunopositive cDNA inserts (hGR1.2) as probe, 12 clones were isolated that, together, covered more than 4.0 kbp. The nucleotide sequences of these clones were determined by the procedure of Maxam and Gilbert⁴⁵ according to the strategy

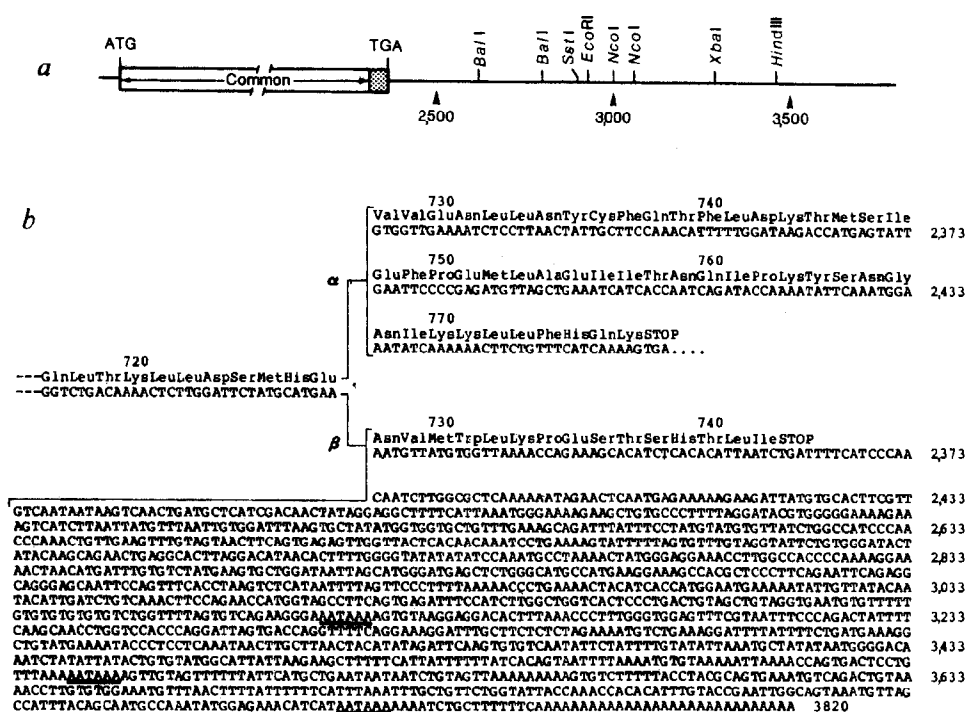


Fig. 3 Restriction map and nucleotide sequence of the 3' end of the human glucocorticoid receptor β cDNA. **a**, The common 6-nucleotide restriction enzyme sites are shown for the 3'-untranslated region of OB10. **b**, The cDNA sequence of the β form (OB10) from nucleotide 2,281 to 3,820 compared with the protein-coding information found in the 3'-terminal coding portion of the α form (OB7). Amino acids encoded by each of the cDNAs are presented above the nucleotide sequences. Putative polyadenylation signals (AATAAA) in the 3'-untranslated sequence of OB10 are underlined.

indicated in Fig. 1a. RNA blot analysis indicated that a cDNA insert of 5–7 kilobases (kb) would be necessary to obtain a full-length clone and sequence analysis indicated that the overlapping clones OB7 and hGR5.16 spanned an open reading frame of 720 amino acids, not large enough to encode the complete receptor. Therefore, a second human fibroblast cDNA library of $\sim 2 \times 10^6$ transformants was screened, yielding a clone (OB10) containing a large insert that extended 150 base pairs (bp) upstream of the putative translation initiation site (see Fig. 1). Sequence analysis predicts two protein forms, termed α and β , which diverge at amino acid 727 and contain additional distinct open reading frames of 50 and 15 amino acids, respectively, at their carboxy termini (see Fig. 1b). The α form, represented by clone OB7, is the predominant form of glucocorticoid receptor because eight cDNA clones isolated from various libraries contain this sequence.

cDNA and protein sequences

Figure 2 shows the 4,800-nucleotide sequence encoding the human α glucocorticoid receptor, determined using clones hGR1.2, hGR5.16, OB7 and OB10. The translation initiation site was assigned to the methionine codon corresponding to nucleotides 133–135 because this is the first ATG triplet that appears downstream from the in-frame terminator TGA (nucleotides 121–123). However, in the absence of amino-terminal peptide sequence information, unequivocal determination of the initiation site is not yet possible. The codon specifying the lysine at position 777 is followed by the translation termination codon TGA. The remainder of the coding sequence is covered by multiple overlapping clones, with OB7 containing a 4.3-kb insert that continues to the poly(A) addition site and OB10 containing the putative initiator methionine. The 3' regions of clones OB7 and OB10 diverge at nucleotide 2,314, as shown by both restriction endonuclease and DNA sequence analysis. At this junction, the α -receptor continues with a unique sequence encoding an additional 50 amino acids whereas the β -receptor continues for only 15 additional amino acids (Fig. 3). The 3'-untranslated region of OB7 is 2,325 nucleotides long, while that of OB10 is 1,433 nucleotides. There is no significant homology between these two regions, as indicated by direct sequence comparison (Figs 2, 3) or by hybridization analysis under stringent conditions (data not shown).

In addition, we have isolated from a human primary fibroblast

library another cDNA clone, OB12 (data not shown), which contains sequences identical to OB7 but uses the polyadenylation signal at nucleotide 3,101 (Figs 1b, 2), giving rise to a shorter 3'-untranslated region. Use of probes specific for the 3'-untranslated region of OB7 to screen a human placenta cDNA library reveals that most clones terminate at the first poly(A) site in OB7. Thus, messenger RNA variation is the apparent consequence of both alternative polyadenylation and alternative RNA splicing (see below). The fact that the human fibroblast library contained both cDNAs suggests that both receptor forms may be present in the same cell.

Analysis of α - and β -receptor protein

Sequence analysis reveals that the α and β forms of the human glucocorticoid receptor are 777 and 742 residues long, respectively; the two forms are identical up to residue 727, after which they diverge. To examine the receptor levels *in vivo*, cytoplasmic extracts from several human and mouse cell lines were probed by immunoblot analysis with a polyclonal antibody directed against the human glucocorticoid receptor³⁷. α - And β -receptor cDNAs were inserted into the SP6 transcription vector to create synthetic mRNA for *in vitro* translation (Fig. 4a). The RNAs were separately added to a rabbit reticulocyte lysate system and the unlabelled products analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The two RNAs programme the synthesis of distinct translation products whose migration differences are consistent with the predicted polypeptide lengths of the two forms (Fig. 4b, lanes 2, 3). Cytoplasmic extracts from untreated IM-9 cells and IM-9 cells treated with 1 μ M triamcinolone acetonide serve as markers (Fig. 4b, lanes 4, 5) for the 94K receptor (the 79K form represents a putative receptor degradation product)⁴⁰. Note that after steroid treatment, the intensity of the 94K band is reduced, corresponding to tighter receptor/chromatin binding and, therefore, receptor translocation to the nucleus. The α form co-migrates with the 94K band of the native receptor while the β form migrates more rapidly (Fig. 4b, compare lanes 2,3 with lanes 4,5). A comparison of cytoplasmic extracts from various human and mouse cell lines reveals the presence of only the α -receptor (Fig. 4b, lanes 6–9). Interestingly, the mouse ADR6 lymphoma cell line⁴⁶, selected for resistance to steroid-induced lysis, contains no steroid-binding activity and shows no immunoreactive receptor (Fig. 4b, lane 7). Therefore, based on characterization of multiple recep-

Fig. 4 Immunoblot comparison of hGR translated *in vitro* with *in vivo* hGR from cell extracts. *a*, The vectors constructed for *in vitro* transcription of the hGR cDNA sequence. The complete α (pGR107) and β (pGR108) coding sequences were placed under the transcriptional control of the SP6 promoter in pGEM1. Vector sequences, noncoding cDNA sequences and coding sequences are indicated by thin lines, thick bars and boxed regions, respectively. The poly(A) tract of ~60 nucleotides is indicated by A_n . Divergent coding sequences are indicated by striped and stippled regions. *b*, Western blot analysis of *in vitro* translation products and cell extracts. Unlabelled translation products synthesized in a rabbit reticulocyte lysate system with no added RNA (lane 1) or with RNA synthesized from pGR108 (β , lane 2) or pGR107 (α , lane 3) were fractionated on a 7.5% SDS-polyacrylamide gel. Additional lanes are: cytoplasmic extracts from IM-9 (lane 4), IM-9 treated with 1 μ M triamcinolone acetonide (lane 5), HeLa (lane 6), ADR6.M1890.AD1 mouse lymphoma (lane 7), S49 mouse lymphoma (lane 8) and EL4 lymphoma (lane 9). Proteins were transferred to nitrocellulose and probed with anti-hGR antibody, followed by 125 I-labelled *Staphylococcus aureus* protein A as described previously⁴².

Methods. To construct an expression vector containing the entire α coding sequence shown in Fig. 2, the 3' coding sequence of OB7 was fused to OB10 5' coding information. OB7 was partially digested with *Eco*RI, completely digested with *Xba*I, and the 1.2-kbp fragment was gel-purified and ligated with *Eco*RI/*Xba*I-digested OB10 to produce the intermediate pOB107. The entire pOB107 cDNA sequence including the 5' poly(G) tract (11 nucleotides, nt) and 3' poly(A) tract (~60 nt) was excised by partial *Pst*I/complete *Bam*HI digestion. The resultant 3.5-kb fragment was gel-purified and inserted between the *Pst*I and *Bam*HI sites of pGEM1 (Promega Biotec) to yield pGR107. Plasmid GR108 was directly constructed from pOB10 by partial *Pst*I/complete *Bam*HI digestion and insertion of the resulting cDNA insert into the corresponding sites of pGEM1. Capped SP6 transcripts were synthesized from *Pvu*II-linearized pGR107 and pGR108, as described by Krieg and Melton⁶², with simultaneous capping effected by reduction of the GTP concentration from 400 to 100 μ M and addition of m⁷GppG (Pharmacia) to 500 μ M. Transcripts were purified by P60 chromatography and translated with micrococcal nuclease-treated rabbit reticulocyte lysate (Promega Biotec) in conditions suggested by the manufacturer. Preparation of IM-9 cytosol from steroid-treated cells was as described previously⁴². Size markers are phosphorylase B (97K), bovine serum albumin (66K) and ovalbumin (45K).

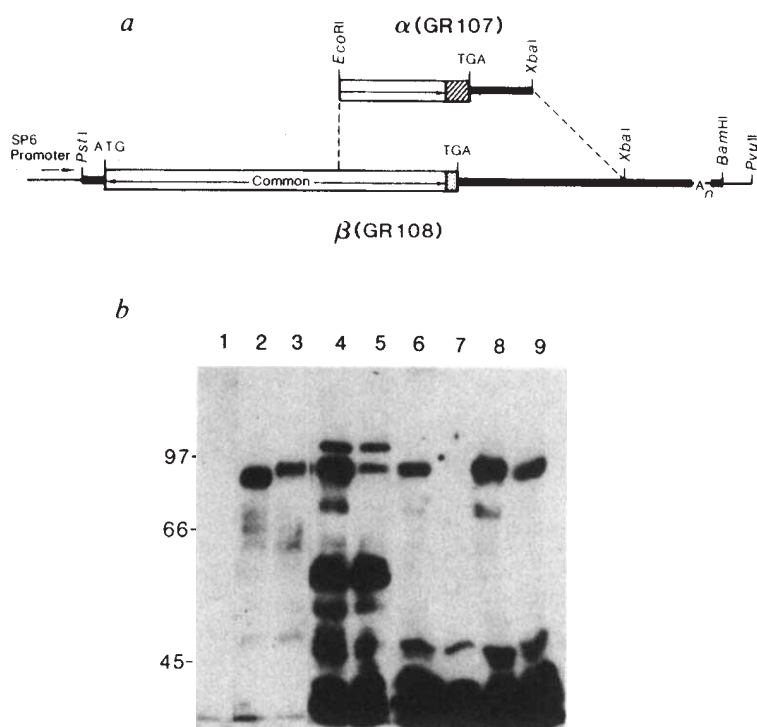
tor cDNA clones and receptor protein by immunoblot analysis, we conclude that the predominant physiological form of the glucocorticoid receptor is the α (94K) species.

Expression of hGR *in vitro*

To provide additional evidence that the cloned receptor is functional, we investigated the possibility that the *in vitro*-translated products might be able to selectively bind corticosteroids. Accordingly, the rabbit reticulocyte lysate was incubated with the radiolabelled synthetic glucocorticoid analogue ³H-triamcinolone acetonide (³H-TA) before or after addition of *in vitro*-synthesized α or β hGR RNA. As shown in Fig. 5, those lysates programmed with α -hGR RNA acquired selective steroid-binding capacity; unexpectedly, the β -receptor synthesized *in vitro* failed to bind competent ³H-TA. The *in vitro*-synthesized α -hGR bound radiolabelled steroid which could be competed with by addition of excess unlabelled cortisol or dexamethasone; however, binding of ³H-TA was not effectively competed with by addition of excess unlabelled oestrogen or testosterone. In contrast, excess progesterone constituted an effective competitor, consistent with the previously reported anti-glucocorticoid activities of progesterone⁴⁷. To confirm these results, the competition experiments were repeated with native glucocorticoid receptor prepared from extracts of human lymphoid cells. Both the *in vitro*-translated receptor and the natural *in vivo* receptor have nearly identical properties with regard to steroid binding and competition with excess unlabelled steroid analogue (Fig. 5).

hGR sequences map to at least two genes

The human glucocorticoid receptor gene has been functionally mapped to chromosome 5. Analysis of somatic cell hybrids constructed by fusing receptor-deficient mouse T cells (EL4)



with human receptor-containing T cells (CEM-C7) indicated that segregants expressing the wild-type CEM-C7 receptor maintained human chromosome 5 while dexamethasone-resistant segregants had lost this chromosome⁴⁸.

To confirm the authenticity of our cDNA clones, we mapped receptor cDNA sequences using Chinese hamster/human somatic cell hybrids containing only human chromosome 5 (HHW454). DNAs extracted from human placenta, HHW454 hybrid cells and Chinese hamster ovary (CHO) cells were digested with *Eco*RI or *Hind*III restriction endonucleases and separated on a 0.8% agarose gel. DNA fragments transferred to nitrocellulose were probed with a portion of the receptor-coding region derived from nucleotides 570–1,640 (hGR1.2A; Fig. 1). In addition to CHO-specific *Eco*RI bands of 6.8 and 17 kbp (Fig. 6a, lanes 2,3), DNA from the hybrid cell line also contains human-specific bands of 3.0 and 5.0 kbp. Unexpectedly, a DNA fragment of 9.5 kbp is found in total human DNA but not in the hybrid line (Fig. 6a, lane 1). Similarly, *Hind*III digestion revealed a 7.5-kbp band that is not present in the chromosome 5 hybrid cell DNA (Fig. 6a, lane 4). These results indicate that the receptor cDNA maps to human chromosome 5, but that there are additional receptor-related sequences elsewhere in the genome. To map these sequences, we used a dual-laser fluorescence-activated cell sorter (FACS) to sort mitotic chromosome suspensions stained with DAPI/chromomycin in conjunction with Hoechst 33258 chromomycin; this technique allows separation of the 24 human chromosome types into 22 fractions⁴⁹. After the chromosomes were sorted directly onto nitrocellulose, the chromosomal DNA was denatured and hybridized to the hGR cDNA probe. In addition to confirming the chromosome 5 localization, additional sequences were found on chromosome 16 (Fig. 6b). To confirm this localization, DNAs from mouse erythroleukaemia cells and a mouse erythroleukaemia cell line containing human chromosome 16 (ref. 50) were digested with

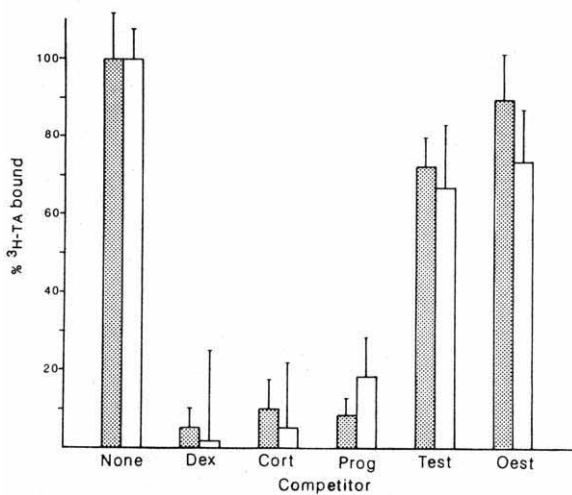


Fig. 5 Steroid-binding of α -hGR (GR107) translated *in vitro*. Binding to IM-9 cytosol extract (stippled bars) and to reticulocyte lysate containing SP6-generated α -hGR RNA (GR107; open bars) are shown. Bars represent bound ^3H -triamcinolone acetonide (TA) determined with a 100-fold excess of various steroid competitors; 100% competition was determined using unlabelled TA as competitor. The values represent the mean of triplicate determinations, with error bars showing $P < 0.05$. Steroid competitors are dexamethasone (Dex), cortisol (Cort), progesterone (Prog), testosterone (Test), and oestradiol (Oest).

Methods. Binding assays were performed in 100 μl containing 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM sodium molybdate, 10 dithiothreitol, 150 mM ^3H -TA (20 Ci mmol^{-1} ; Amersham) and 10 μl translation mixture or 100 μg fresh IM-9 cytosol. Unlabelled steroid competitor (15 μM) was added as indicated. After 2 h at 0°C , samples were extracted twice for 5 min each with 5 μl of 50% dextran-coated charcoal to remove unbound steroid, and counted. Uncompeted and fully competed values for the α glucocorticoid receptor (GR107) were 490 and 290 c.p.m., respectively. Reticulocyte lysate translation mixtures without added transcript or programmed with β -receptor SP6 RNA (GR108) contained no competeable ^3H -TA binding.

*Hind*III and probed with hGR cDNA (Fig. 6c); as predicted, the only DNA fragment found in the hybrid and not in the control was the 7.5-kbp DNA fragment, thus establishing the chromosome 16 assignment (Fig. 6c, lanes 1, 2).

Additional Southern blot analyses using the *Eco*RI-*Xba*I fragments from OB7 and OB10 3'-untranslated regions revealed hybridization only to chromosome 5 (data not shown). We conclude that both the α - and β -receptor cDNAs are probably encoded by a single gene on chromosome 5 and suggest that the two cDNA forms are generated by alternative splicing. In addition, we conclude that another gene residing on human chromosome 16 contains homology to the glucocorticoid receptor gene, at least between nucleotides 570 and 1,640. It is not clear whether these sequences on chromosome 16 represent a related steroid receptor gene, a processed gene or pseudogene, or a gene that shares a common domain with the gene for the glucocorticoid receptor. Genomic cloning and DNA sequencing may provide the answer.

To determine the size of the mRNA encoding the glucocorticoid receptor, Northern blot hybridization³¹ experiments were performed using cytoplasmic mRNA isolated from a human fibroblast cell line, HT1080. Using the hGR1.2 coding sequence as probe, multiple mRNAs of 5.6, 6.1 and 7.1 kb were detected. Treatment of these cells with glucocorticoids for 24 h leads to a 2-3-fold reduction in receptor mRNAs, suggesting a potential negative feedback regulation.

Discussion

Structural analysis of the glucocorticoid receptor is a prerequisite for gaining insight into the mechanisms by which this regulatory molecule exerts its effects on gene transcription. Here, we have presented the primary sequence of the human glucocorticoid receptor deduced from nucleotide sequence analysis of cDNA clones.

Isolation of hGR cDNAs has revealed the existence of multiple mRNAs encoding at least two forms of the polypeptide. The predicted proteins differ at their carboxy termini by the substitution of 50 amino acids in the case of α -hGR and 15 amino acids in the case of β -hGR. The α glucocorticoid receptor is the major

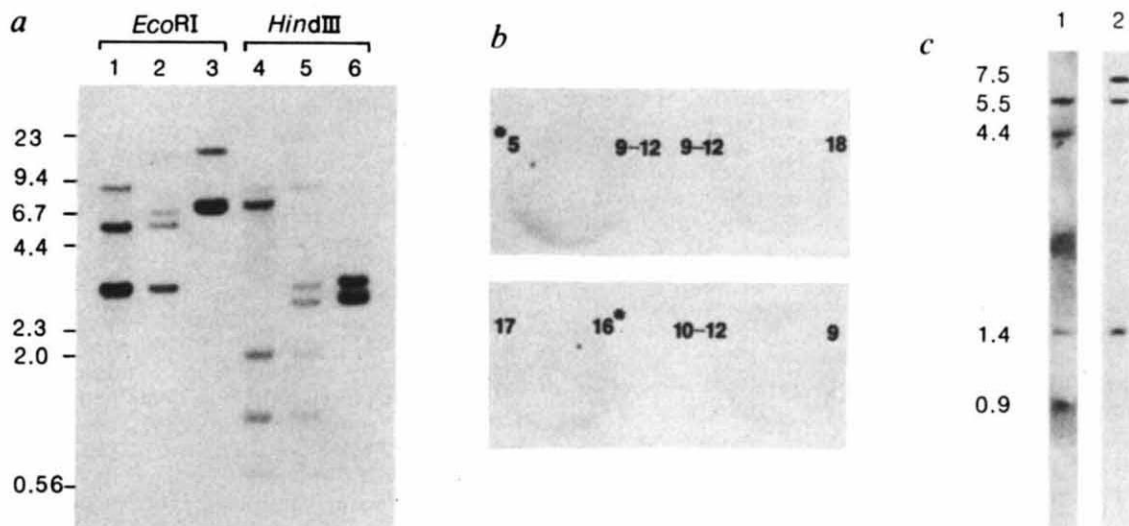
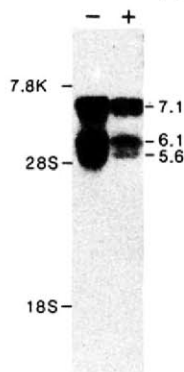


Fig. 6 Chromosome mapping analysis of hGR cDNA. **A**, 10 μg of DNA from human placenta (lanes 1, 4), CHO/human somatic cell hybrid (HHW454, lanes 2, 5) containing chromosome 5 as its only human complement, or CHO (lanes 3, 6), was digested with *Eco*RI (lanes 1-3) or *Hind*III (lanes 4-6) to completion, fractionated on a 0.8% agarose gel and transferred to nitrocellulose paper. **B**, Chromosomes (3×10^4) prepared from a human lymphocyte cell line, stained with 4,6-bis(2"-imidazolyl)-4H,5H)-2-phenylindole (DIP)/chromomycin A3 and sorted using a dual-laser custom FACS IV chromosome sorter⁶³, were denatured and neutralized on nitrocellulose paper. Note that Hoechst/chromomycin-stained chromosome 9 was sorted with chromosomes 10-12. **C**, 10 μg of DNA from the parental mouse cell line MEL (lane 1) or the parentally derived somatic cell hybrid carrying human chromosome 16 (ref. 50; lane 2) was digested with *Hind*III and separated on a 0.8% agarose gel, then transferred to nitrocellulose paper. All filters were probed with the 1,100-bp insert from hGR1.2, nick-translated to a specific activity of 3×10^8 c.p.m. μg^{-1} and hybridized in $5 \times \text{SSPE}$, $1 \times \text{Denhardt's}$, 0.1% SDS, 50% formamide, 100 $\mu\text{g ml}^{-1}$ denatured salmon sperm DNA, 50% dextran sulphate at 42°C for 18 h. Filters were washed twice (for 30 min each) in $2 \times \text{SSC}$ at 68°C and exposed to X-ray film at -70°C with an intensifying screen.

Fig. 7 Northern blot analysis of hGR mRNA. 10 μ g of poly(A)⁺ mRNA from human HT1080 fibroblast cells, collected after 24 h without (–) or with (+) treatment with 10 μ M dexamethasone, was electrophoresed through a 0.8% agarose/1% formaldehyde gel, stained with acridine orange (120 μ g ml^{–1}) and transferred to nitrocellulose. The filter was hybridized overnight with nick-translated hGR1.2 (10⁶ c.p.m. ml^{–1}, specific activity 10⁸ c.p.m. μ g^{–1}) and washed with 2 $\times$ SSC at 68 °C. Sizes were estimated from human fibronectin mRNA (7.8 kb), and 28 S (5.0 kb) and 18 S (2.1 kb) ribosomal RNAs.



form identified in several human cell lines and cDNA libraries. However, a recent report by Northrop *et al.* characterizes two forms of the receptor in mouse lymphoid cells⁵². The relationship of α - and β -hGR to the mouse doublet species remains to be established. Also, the cellular distribution and potential function of β -hGR are unclear, although it is possible that variant receptors are used for tissue-specific functions. We are now generating antisera to synthetic peptides specific for each human receptor form to elucidate their tissue-specific expression.

Among the cDNAs selected using the immunopositive phage DNA insert hGR1.2A as probe were those containing 3' ends similar to OB7, except that polyadenylation was signalled earlier by the use of an AATAAA at nucleotide 3,101. These clones have been isolated from both human fibroblast and placental libraries (data not shown). Alternative poly(A) site selection is a feature of many eukaryotic transcription units⁵³. In some instances, selection of poly(A) sites specifies particular polypeptide products^{54–57} while in other cases, alternative poly(A) site selection produces no change in the primary structure of the polypeptide⁵⁸. The selection of poly(A) sites during receptor transcription may (1) alter the stability of the mRNA in a particular tissue, (2) lead to splicing changes, or (3) be random, with no physiological consequence.

The *in vitro* translation studies described here provide direct evidence that the cloned molecule encodes the complete glucocorticoid receptor. First, the *in vitro*-translated product is identical in size to the native glucocorticoid receptor and is immunologically reactive with receptor-specific antiserum.

Second, the *in vitro*-translated protein acts functionally as a glucocorticoid receptor in that it is capable of selectively binding the synthetic glucocorticoid triamcinolone acetonide. This binding is specifically competed with by glucocorticoids, glucocorticoid analogues and progesterone but is not competed with by the sex steroids testosterone and oestrogen. In this respect, the *in vitro*-translated receptor behaves identically to the *in vivo* receptor from human lymphoid cells, providing the first evidence for a function of the cloned molecule. The acquisition of steroid-binding properties does not appear to require any specific modifications or, if it does, these modifications can occur in the *in vitro* translation mix.

The results presented here provide the information necessary for studying the molecular interactions of a eukaryotic transcriptional regulatory protein with its target genes. These structural studies provide a basis from which the glucocorticoid receptor, its gene, and its RNA products can be analysed. Furthermore, the ability to express receptor *in vitro* provides a novel means by which the consequence of specific *in vitro* mutagenesis can be rapidly tested.

In addition to the *in vitro* studies, the analysis of several existing rodent cell lines^{49,59–61} with well-characterized receptor defects in both the DNA- and steroid-binding domains should facilitate future analysis. Furthermore, the isolation of genes responsive to glucocorticoids and specific regulatory elements by both mutagenic and protein-binding studies suggests that this protein will serve as a very useful model for analysis of inducible eukaryotic gene regulation.

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A 12.6-ms pulsar in Cygnus X-3

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The X-ray binary Cygnus X-3 has long been suspected of containing a fast, young pulsar, but previous observations at various wavelengths have failed to provide any evidence of a signal with a periodicity of less than 4.8 h (the well-known periodicity in the X-ray and infrared wavebands which is ascribed to orbital motion of a binary system). Here we report measurements of 1,000-GeV γ -rays which suggest a pulsar period of 12.5908 ± 0.0003 ms, for data recorded in 1983 at the times of X-ray maximum in the 4.8-h cycle. The conservative probability of such a periodicity occurring by chance is 4×10^{-7} and, moreover, supportive evidence is provided by some earlier data.

Cyg X-3 has been observed many times by γ -ray telescopes at TeV energies¹⁻⁵ and more recently by cosmic-ray detectors at PeV energies⁶⁻⁷. There is a consensus that the TeV emission is confined to two short periods in the 4.8-h X-ray modulation cycle⁸, with the stronger period occurring about X-ray phase 0.625. Previous measurements using the Dugway array of four telescopes⁹ have indicated that this emission may last for only ~ 10 min. Until 1983, the gross counting rate of the telescope array was insufficient to allow a search for a possible periodicity.

Observations using improved detectors were made in August–October 1983, with Cyg X-3 being continuously tracked for long intervals under a wide range of weather conditions. The best atmospheric conditions were encountered during a 6-h observation made on 1983 September 12, with ideal weather. The time of arrival of each event was recorded with a resolution of 1 μ s and an absolute uncertainty of ~ 0.5 ms. Each event time was adjusted to Ephemeris time at the solar system barycentre using the MIT ephemeris². At 0519 UT the 4.8-h X-ray phase was 0.625. At this phase a count rate excess was noted which lasted for ~ 7 min. The most important feature of this 7-min interval is that it offers, for the first time, a sample of sufficient size and signal strength to make a pulsar period search worthwhile.

We have tested the 450 events contained in the 7-min interval for periodicity over the range 10 ms to 50 s, using the most appropriate test for the power in the fundamental—the Rayleigh test¹⁰. The period with by far the smallest probability of a chance origin was 12.5908 ms, the probability being 4.8×10^{-8} (see Fig. 1a). Allowing for the number of periods tested, the true significance of this periodicity is 3.3×10^{-3} or 3σ . Although in itself not particularly significant, this possible periodicity has the support of further independent evidence.

First, we have considered the possible correlation between counting rate and periodicity within the 7-min interval. If the periodicity is genuine, the onset and decay of the count rate excess should, on average, be mapped by the variation in the fraction of the counts which are periodic. In the null case (that the apparent periodicity is just a random fluctuation) the fraction of counts which are apparently periodic should be independent of the actual number of counts, hence there should be no correlation between these values. As the joint probability of a segment of the 7-min interval having a particular fractional periodicity and also having a particular gross number of counts (given the null hypothesis of no real periodicity) can be factorized, a correlation would provide independent evidence of periodicity¹¹. Although, formally, product moment correlation requires normally distributed samples, in practice a wide variation of sampling distributions is found to be permitted^{12,13}. We find that the strength of the 12.5908-ms periodic component

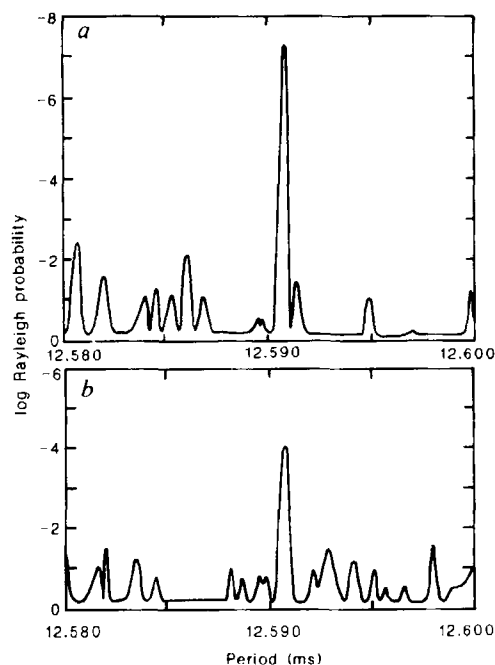


Fig. 1 a, The chance probability for periodicity in the 450 events containing the count rate excess on 1983 September 12, as a function of trial period. b, The chance probability of periodicity for the events on 1983 October 2 at precisely the same phase in the 4.8-h cycle as was investigated on 1983 September 12, as a function of trial period.

correlates (at a significance level of 9.5×10^{-4} or 3.1σ) with the count rate in independent 1-min data segments within the 7-min interval. The correlation arises mainly from two of the minutes (which are 3 min apart and are roughly centred in the 7-min interval) during which both the counting rate and the periodic signal are both particularly strong.

Second, we examined other nearby data sets containing precisely the same phase in the 4.8-h period. During August–October 1983, seven other such observations of Cyg X-3 were made. In no case were the climatic conditions stable enough to allow reliable use of the minute-by-minute count rates to unambiguously identify a count rate excess. However, tests for millisecond periodicity at a known period are possible in such data. For one of the seven observations (1983 October 2) exactly 100 4.8-h cycles after the detection on September 12, a periodicity was observed in the 7-min interval at 12.5908 ms, with a chance probability of 4×10^{-4} (see Fig. 1b) (the larger chance probability reflects the lower counting rate due to non-ideal climatic conditions during the October measurements).

The interpretation of this second occurrence of periodicity is complicated by the possible presence of two 19-day periodic effects: a 19.3-day amplitude modulation⁵ of the TeV emission at X-ray phase 0.625 based on our 1981/82 data, and the 18.7 day phase modulation of amplitude ± 3 min in the time of the X-ray minimum found by the COS-B X-ray detector¹⁴ and attributed to apsidal motion. If the 19.3-day amplitude modulation is genuine, and since the excess on September 12 was near a 19.3-day peak, the October 2 observation would be the one expected to show the most significant periodic excess. Because of the 18.7-day phase modulation, the 20-day separation of our two observations may produce up to a minute in phase difference. We note that data in a 7-min interval centred 50 s later than the precise time predicted from the X-ray ephemeris and our previous measurement, show periodicity at 12.5908 ms with a smaller chance probability, that is, 4×10^{-5} .

To be conservative, we have (1) allowed degrees of freedom for all seven observations, ignoring any possible 19.3-day amplitude modulation (although this is noted for future confirmatory observations) and (2) ignored the possible improvement in

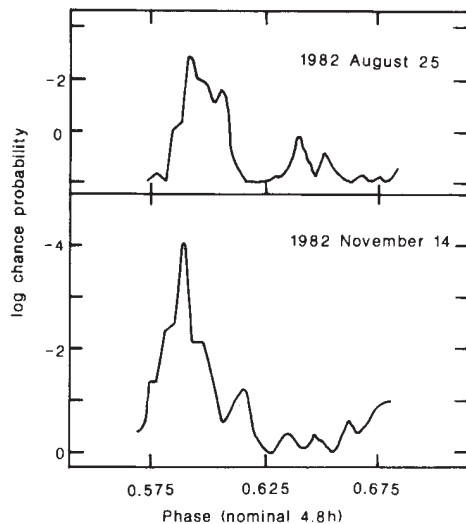


Fig. 2 The chance probability of periodicity (12.5908 ms) as a function of phase in the nominal 4.8-h period, for observations on 1982 August 25 and November 14.

chance probability by shifting the time of the 7-min interval to allow for possible apsidal motion. This leads to a chance probability of 2.8×10^{-3} for detecting a 12.5908-ms periodicity on any one of the seven occasions.

Finally, we have demonstrated previously that those events which trigger two separate telescopes spaced 60 m apart (twofold events) have a narrower acceptance angle distribution than is the case for events registered in a single telescope¹⁵. A point source of γ -rays centred in the field of view will consequently have a proportionally greater effect on the rate of cosmic-ray background detected as twofold responses than is the case for single-telescope responses. In the present observation, the fraction of the twofold events showing periodicity was 1.5 times greater than that of the single-telescope events, in accord with our previous experience. However, the restricted sample size gives an uncertainty of ± 0.5 in this value so that, in this case, formal use cannot be made of the increased sensitivity of the two-fold responses.

Although the general periodicity search was terminated at 10 ms because of the possible loss of coherence for shorter periods, periods near integral factors of 12.5908 ms have been searched for in shorter segments of the 7-min interval. No evidence was found to indicate that 12.5908 ms is a multiple of a shorter periodicity.

Our data for 1983 thus provide chance probabilities of: (1) 3.3×10^{-3} (September 12 periodicity allowing for all periods tested); (2) 9.5×10^{-4} (correlated periodicity and count rate on September 12); and (3) 2.8×10^{-3} (periodicity on October 2 at precisely the predicted phase in the 4.8-hr cycle, allowing for non-detection of signals from six other data sets). These three probabilities are derived from independent data or independent tests on the same data and may therefore be combined. The combined probability of 4×10^{-7} is conservative.

To check for possible instrumental effects, off-source data from the same and adjacent nights were analysed similarly and the Rayleigh test probability distributions are found to be those expected by chance, as is the distribution during the 7-min excess, discounting the 12.5908-ms peak. The 12.5908-ms periodicity was detected in each of the four well-separated (100 m) telescopes which were operated and triggered independently, largely excluding the possibility that local pick-up caused the increase in counting rate. Also, more data were available on each event other than the time of arrival (UT). The relative times of triggering of each detector were recorded with 150-ps resolution. For those events which triggered two or more detectors, the correct time separation was observed for Cerenkov light flashes in the field of view. The pulse height in all three of the photomultipliers on each detector was also recorded for each

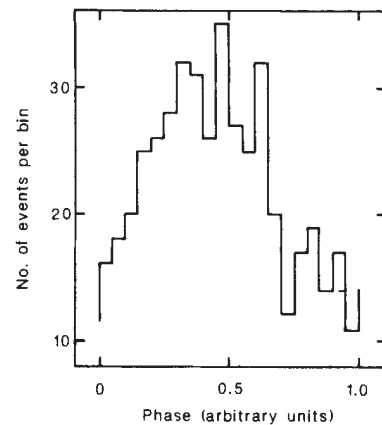


Fig. 3 Light curve for the TeV γ -ray emission during the 7-min data set at X-ray maximum on 1983 September 12.

event, and the spectrum during the 7-min interval was that expected for Cerenkov flashes. All the anode currents and photoelectron noise trigger rates of the individual photomultipliers were also recorded and were normal during the 7-min interval. Therefore, we believe that the events giving rise to the periodic excess were genuine Cerenkov light flashes in the field of view.

We have examined our 1982 data for instances of similar periodicity. In 1982 the counting rates were considerably lower than in 1983, and we would not expect any significant contribution to the overall chance probability of periodicity from an interpretation of these data. Furthermore, the absolute phase in the 4.8-h orbit of the data to be tested will have uncertainty due to variations arising from possible apsidal motion and from errors in the ephemeris for the 4.8-h period. The uncertainties in the 4.8-h period suggest variations of ~ 5 –10 min in the phase in 1982 relative to that in 1983. We have therefore passed a window of 7 min duration through the data, incrementing the start time by 1-min steps spanning ± 15 min from the nominal phase 0.625. The nominal 4.8-h orbit uses the van der Klis and Bonnet-Bidaud⁸ ephemeris incorporating a period derivative. Each data set was tested for periodicity at 12.5908 ms. There is evidence for periodicity during 2 of the 14 observations (compared with 2 out of 8 in 1983). The strength of the periodicity as a function of the nominal 4.8-h phase on these two occasions is shown in Fig. 2. These two detections concur in the emission being earlier than the nominal phase. There is thus support for 12-ms periodicity from the 1982 data. Our 1981 data were taken in drift-scan mode with detectors of lower counting rate and less precise millisecond timekeeping and thus are less suitable for this analysis.

If the 4.8-h X-ray modulation is due to an orbit, as is generally concluded, the sections of data suggesting periodicity and lasting for 7 min should show some evidence of an orbitally derived period derivative. The periodicity test has been relaxed for a range of values of period derivative between -10^{-8} and $+10^{-8} \text{ s s}^{-1}$. The combined probability for the two positive data sets in 1983 was found to minimize for a small negative derivative: $-1.2 \pm 0.7 \times 10^{-9} \text{ s s}^{-1}$. A similar treatment of the two data sets in 1982 gave a derivative of $-0.6 \pm 0.4 \times 10^{-9} \text{ s s}^{-1}$. These estimates combine to give $-7.5 \pm 3.5 \times 10^{-10} \text{ s s}^{-1}$. This negative value of derivative places the source of the TeV emission, which coincides with the X-ray maximum, in the rear half of the orbit. Because the TeV emission is at X-ray maximum, this result (which is significant only at the 2σ level) conflicts with the two main models for the X-ray modulation—the cocoon model and the stellar wind model. In these models the X-ray emitter is in front of the companion at X-ray maximum. Both models assume the X-ray source is an accreting neutron star or a pulsar and the X-ray modulation is due to variations in the absorption or scattering, and both models require a significant orbital eccentricity. However, the sign of the derivative is consistent with a TeV-emission model¹⁶ and places the source of the very-high

energy ions behind the companion's atmosphere at the time of TeV emission. With the assumptions that TeV γ -ray emission occurs when the pulsar is almost directly behind the companion, and that the orbit is nearly circular (as inferred from the COS-B observations of the 18.7-day X-ray phase modulation), the observed period derivative of $-10^{-9} \text{ s s}^{-1}$ would correspond to an orbital velocity of $\sim 80 \text{ km s}^{-1}$. In this case the Keplerian mass function would be $\sim 0.01 M_{\odot}$.

Recently, two models have been proposed to explain the production of TeV and PeV γ -rays from Cyg X-3: (1) the luminosity is derived from the rotational energy of a rapidly spinning ($\sim 10 \text{ ms}$) neutron star of high magnetic field¹⁶, and (2) acceleration of ions occurs in an accretion disk surrounding a neutron star by the unipolar inductor mechanism¹⁷. Both models require the high-energy ions to interact with the atmosphere of the companion to produce the γ -rays. The light curve for the TeV emission during the 7-min interval on 1983 September 12 (for a period of 12.5908 ms and including the period derivative) is broad (see Fig. 3). The curve for the emission on October 2 is similar, and both curves have the same form as the TeV light curves of other X-ray binaries (Hercules X-1¹⁸; 4U0115¹⁹) and the 6-ms pulsar PSR1953+29²⁰. This is in contrast to the very sharp light curve in the case of an isolated pulsar—the Crab²¹. This suggests that accretion from a pulsar's binary companion plays an important part in the TeV emission.

The TeV γ -ray flux appropriate to this observation is consistent with our previous measurement¹ of $10^{-39} \text{ W m}^{-2} \text{ Hz}^{-1}$. The differential power-law spectral index of ~ 1 implicit in a comparison of this flux with the X-ray flux suggests that TeV γ -rays constitute the majority of the electromagnetic emission from Cyg X-3. The total energy flux, over all wavelengths, has been estimated at $\sim 10^{38} \text{ erg s}^{-1}$; this, together with a pulsar period of 12.5908 ms, would require a spin-down period derivative, given a magnetic field similar to that of the Crab pulsar, of about $10^{-14} \text{ s s}^{-1}$. On the other hand, an accretion-driven low-magnetic-field system may be in equilibrium or may be spinning up. In either case, the change in period expected between the observations in 1983 and those in 1982 would be $< 0.3 \mu\text{s}$. Our sampling uncertainty in period is about the same, precluding any measurement of a secular period change until a precise orbit has been determined.

Thus, we have *prima facie* evidence from our 1983 data, at a chance probability of 4×10^{-7} , that the excess counts of 1,000-GeV γ -rays noted from Cyg X-3 at orbital phase 0.625 in the 4.8-h X-ray cycle are all periodic, with a period in the orbit in 1983 September/October of $12.5908 \pm 0.0003 \text{ ms}$. Other qualitative evidence from observations in 1983 and 1982 supports this suggestion. However, further observations of Cyg X-3 are required to confirm the existence of this periodicity. Note that for all other wavelength bands where Cyg X-3 has been detected, current models would predict that such a millisecond pulsar periodicity would be greatly attenuated due to dispersion or scattering in the cocoon or dense stellar wind surrounding the companion. However, rapid quasi-periodic oscillations reported recently²² from GX5-1 have been interpreted^{22,23} as beating between the period of a fast pulsar and the orbital period of the inner edge of the accretion disk. Such indirect effects of a 12.59-ms pulsar may be detectable, or may be involved in the longer-period quasi-periodic oscillations already detected from Cyg X-3 (ref. 24).

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Optical polarization observations of circumsolar dust during the 1983 solar eclipse

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It is very difficult to make optical observations from the Earth where the angular distance from the Sun is greater than $3 R_{\odot}$ (where $R_{\odot}$ is the angular radius of the Sun) because of the large and uncertain correction for sky radiation. To overcome these difficulties, we have now made optical polarization observations at four wavelengths (5,330 Å, 5,970 Å, 7,200 Å, 8,015 Å) for the outer solar corona at an altitude of 30 km, using a balloon-borne silicon-intensified (SIT) TV camera during the total solar eclipse on 11 June 1983 in Indonesia. As reported here, a polarization excess at an elongation between 4 and $5 R_{\odot}$ (1° and 1.25°) from the Sun in the ecliptic plane was observed at a wavelength of 8,015 Å on the two-dimensional frame. This is interpreted as an enhancement of dust grains distributed in a ring around the Sun approximately in the ecliptic plane.

Infrared excesses have been observed^{1–5} at the elongation around 4 and $10 R_{\odot}$ which have confirmed the existence of a

Table 1 Polarization distribution at the region of enhancement

θ/R	3.6	3.8	4.0	4.2	4.4	4.6	4.8	5.0	5.2	5.4
South										
16	15	14	14	13	11	10	8	9	8	8
14	14	15	14	14	12	11	9	8	6	6
12	14	16	15	14	12	11	10	8	7	6
10	14	17	16	14	13	11	11	9	6	6
8	14	17	17	15	14	11	10	9	6	6
6	14	17	17	16	15	11	9	9	7	6
4	12	17	18	17	14	13	10	9	8	7
2	12	18	19	17	15	13	13	9	8	7
North										
0	12	18	19	16	14	14	12	9	8	8
2	12	17	19	17	15	14	11	10	8	7
4	0	17	18	18	16	14	12	9	8	8
6	0	17	19	17	15	14	13	10	9	8
8	0	17	20	18	16	14	12	10	9	7
10	0	16	20	17	16	14	12	10	8	8
12	0	15	19	16	15	11	11	11	8	7
14	0	14	19	17	16	15	11	10	8	7
16	0	13	19	17	15	14	10	10	7	6

R is the angular distance from the Sun in units of $R_{\odot}$. θ is the angle of radial line from the Sun to the ecliptic. $\theta = 0^{\circ}$ is on the ecliptic and $\theta = 10^{\circ}$ in the north is on the solar equator. At $R = 3.6 R_{\odot}$, data are probably affected by the occulting disk.

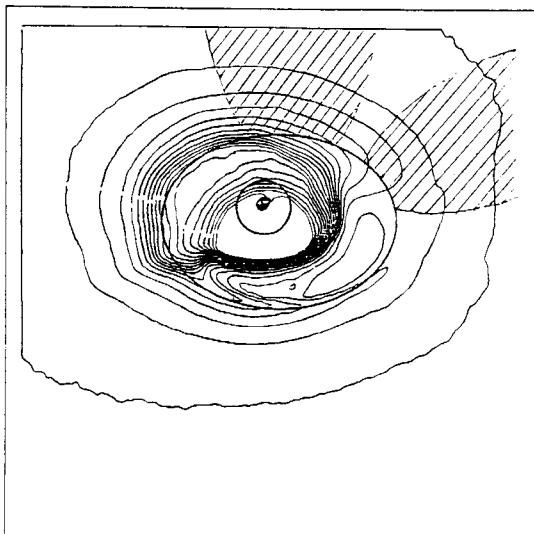


Fig. 1 An intensity contour map at a wavelength of 8,015 Å. The small circle and the large ellipse are the positions of the Sun and the occulting disk, respectively. The two areas suffering ghost images are hatched. The contour unit is arbitrary. The field-of-view is $5^\circ \times 5^\circ$ corresponding to $20 \times 20 R_\odot$. North is down and west is left.

dust-free zone inside the solar distance (radial distance from the Sun) at about $4 r_\odot$ ($r_\odot$ is the linear radius of the Sun), and indicates that there is a ring-shaped enhancement in the density of dust grains along the outer edge of this zone. To observe these areas, we used an optical telescope with a field of $5^\circ \times 5^\circ$ ($20 \times 20 R_\odot$) centred almost on the Sun. Because our SIT TV camera has 256×256 pixels, the observed resolution is $0.08 R_\odot$. Apart from the introduction of a small shift of the Sun (10 arc min to the south-west; upper left in Fig. 1) from the centre of the occulting disk to eliminate bright light from the inner corona, the observations were successful. Unfortunately, the shift had severe saturation effects on the observations at 5,330 Å, 5,970 Å and 7,200 Å, and therefore only the observational data at 8,015 Å are presented here. All the data, including those at the other wavelengths, will be published elsewhere^{6,7}.

During the 140 s of the observation, we rotated two wheels: the filter wheel was kept fixed for 15 s (in each filter) while the polarizer wheel was rotated to each of the eight positions, remaining in each position for 1.8 s. We thus have two complete sets of polarization observations at the four wavelengths. All observational data were recorded by a video recorder on-board the balloon. After recovery of the tape, all video data were transferred to digital data using a computer with a 16-bit double-frame memory. To control for the non-uniformity of the detector sensitivity of the SIT TV cameras and the instrumental polarization created by our telescope system, artificial uniform unpolarized light was measured. After correction, the error of polarization at each point of the detector was estimated to be $\pm 1\%$. Although the asymmetrical position of the Sun in the TV frame made two ghost images (see Fig. 1), there was no effect on the other areas in the field.

Figure 2 shows a contour map of the degree of polarization, where the length and direction of each line denote the degree of polarization and the direction of the electrical vector, respectively. The polarization vector should be perpendicular to the radial direction from the Sun, because coronal light is scattered by coronal electrons (K corona) and interplanetary dust (F corona). Most vectors shown in Fig. 2 satisfied this condition. The numerical values of the degrees of polarization in the areas of interest are shown in Table 1 and indicate a smooth variation of polarization between successive points. It has been difficult to separate the two components of the F and K corona in the observed intensity, for the only way to separate them is to

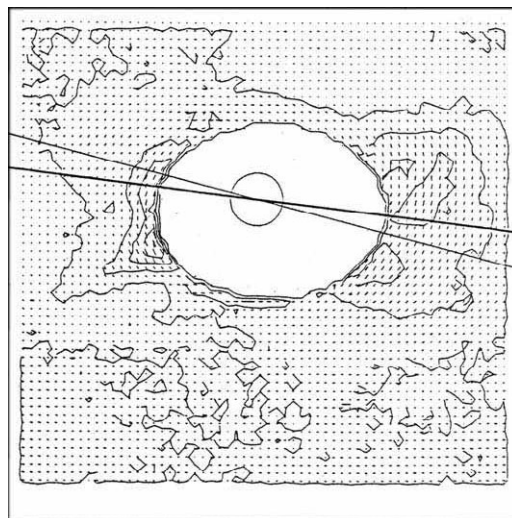


Fig. 2 A contour map of polarization degree in a field of $5^\circ \times 5^\circ$. Contour unit is 5%. The ecliptic plane and solar equator are shown by thick and thin lines, respectively. North is down and west is left. The length and direction of each short line denote degree of polarization and direction of electrical vector, respectively. Note that there is some effect of the ghost images in the areas shown in Fig. 1.

observe the depth of Fraunhofer lines in the spectrum of the corona and to compare them with the depth in the solar spectrum⁸. We have observed the total intensities of the combined F and K coronal components and so could obtain no information on their relative proportions. Van de Hulst⁹ collected data on the F and K coronal components between 1 and $10 R_\odot$ and showed that the K component is brighter than the F component at an elongation of $< 2 R_\odot$, but decreases to $< 20\%$ of the F component beyond an elongation of $3.5 R_\odot$ except for the area of the coronal stream. Figure 2 shows clearly an area with a high degree of polarization which corresponds to a coronal stream.

We have compared our polarization distribution in the ecliptic plane with other results. Blackwell and Petford⁸ observed the outer corona and obtained the intensity and polarization of the F and K components at an elongation greater than $5 R_\odot$. Following their results, the degree of polarization of total light for the F and K components is $< 8\%$. During the solar eclipse on 5 February 1962, Saito and Hata¹⁰ made an observation of the polarization at 6,200 Å and showed that the degree of polarization decreases between 1.2 and $3.0 R_\odot$ in elongation and that the extrapolated value at an elongation of $5 R_\odot$ is $< 10\%$. Our results show $\sim 17\%$ polarization at an elongation of between 4 and $5 R_\odot$ (see Fig. 3). It is not clear why the previous results did not show high polarization at elongations between 4 and $5 R_\odot$, but we think that photographic observations of polarization such as those of Saito and Hata have a low signal-to-noise ratio at the elongation considered and that photoelectric observations of polarization cover only a small coronal area in each limited observational period during the solar eclipse. Unfortunately, Blackwell and Petford's extensive observations were carried out at large elongations ($5\text{--}16 R_\odot$). Our observations were carried out using a SIT TV camera with as high an accuracy as is obtained with photoelectric observations and, using a two-dimensional frame for the photographic observations, show an area of high polarization (see Figs 2, 3).

If there is an enhancement in the density of dust grains around $4 r_\odot$ as suggested by the infrared observation, the enhanced grains located between 4 and $5 r_\odot$ on the line-of-sight, at an elongation between 4 and $5 R_\odot$, scatter the solar light with a scattering angle of around 90° . The scattered light is polarized by 0–60% depending on the refractive index and radius of dust grains and on the wavelength of the light^{11,12}. Therefore, one

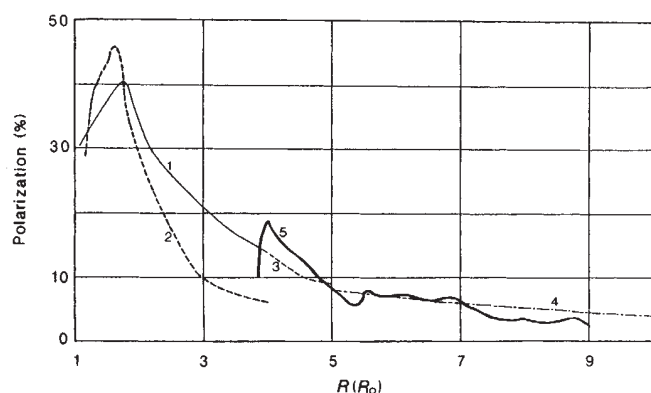


Fig. 3 Degree of polarization depending on the elongation in the ecliptic plane. Thin solid (1) and thick dashed (2) lines are those observed by Saito and Hata¹⁰ in the east and west directions respectively. Thin dashed line (3) is an extrapolation of thin solid line. Dashed-dotted line (4) shows the results of Blackwell and Petford⁸. Thick solid line (5) is our result in the west direction which shows a high degree of polarization at the elongation between 4 and 5 $R_{\odot}$. There is a jump of polarization from 0 to 15% at the elongation of 3.8 $R_{\odot}$ corresponding to the cutoff point of the occulting disk, but the peak of polarization at 4.0 $R_{\odot}$ is real. The observational error is 1% at the inner region.

can expect a high value for polarization at an elongation between 4 and 5 $R_{\odot}$ in the ecliptic plane as a result of dust grains that have a scattering angle of $\sim 90^\circ$. Because there is no enhancement of polarization in the direction perpendicular to the ecliptic plane in Fig. 2, the region with the highest number density of dust grains does not extend far from the ecliptic plane.

We conclude that there is an enhancement of the degree of optical polarization at an elongation between 4 and 5 $R_{\odot}$ which suggests that the number of dust grains distributed in a ring around the Sun increases approximately at the ecliptic plane.

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Decay of the cometary bow shock

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Cooling processes will weaken the bow shock that is expected to form when the solar wind plasma encounters a gassy comet. As the supersonic wind penetrates the comet's outer coma, accreting freshly ionized cometary atoms and molecules, it needs a shock to adjust to the inner subsonic conditions. The cometary ions, implanted in the plasma stream, are accelerated by the associated fields and take up much of the decrease in streaming energy. The

subsonic flow in comets is distinguished by strong cooling, effected primarily through ion-molecule reactions between the energetic implanted ions and the neutral gas coma. We argue here that such cooling can cause complete decay of the shock's flanks, as probed by the ICE (International Cometary Explorer) spacecraft at comet Giacobini-Zinner.

To the on-rushing solar wind, the extensive and rarefied cometary coma presents a very different obstacle from a planetary magnetosphere, or even a planetary exosphere such as Venus. The solar wind plasma penetrates freely through the outer coma, except that associated electric and magnetic fields couple it to occasional freshly ionized cometary atoms and molecules. These implanted cometary ions have commonly been viewed as 'picked up' in the plasma flow, their bulk effect being modelled as sources of mass m , momentum S and energy E added to the gas-dynamic equations for the flow¹. The sources combine to constitute the generalized drag^{2,3}

$$D = \frac{1}{2}(\gamma + 1)m - \gamma S/u + \frac{1}{2}(\gamma - 1)E/u^2 \quad (1)$$

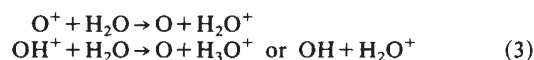
in a flow at speed u , specific heat ratio γ . Evidently, mass-accreting ($m > 0$) and frictional ($S < 0$) contributions to D are offset by cooling ($E < 0$). This drag coefficient D enters the 'throat' equation, crucial for investigating transonic gas-dynamic flows⁴: for a streamtube of cross-sectional area A and length z ,

$$(1 - M^2)du/u = M^2 D dz / \rho u - dA/A \quad (2)$$

where $M = \sqrt{(\rho u^2 / \gamma p)}$ is the Mach number, ρ the density and p the pressure. While flows with 'mass-loading' alone with D everywhere positive have attracted much attention, the presence of an inner region with negative D (see ref. 5) is emphasized here.

Solutions investigated in the case $S = 0$, $E = 0$, have subsonic flow ahead of the nucleus that is close to incompressible and possesses a weak bow shock^{5,6}. The shock is about Mach-2 at the nose and is very blunt, crossing $z = 0$ at some 2.5-3 times the stand-off distance; its Mach number increases towards the flanks⁷. Its strength is sufficient to diverge the flow ahead of any impenetrable inner region (magnetosphere/ionosphere), which would be smaller in size by an order-of-magnitude (for computational purposes, Biermann *et al.*⁷ assumed an artificially large inner cylinder, while Lipatov's^{8,9} magnetohydrodynamic calculation has an inner magnetic sheath conditioned by large gyroradius effects).

However, momentum and energy losses are likely to be very significant. Implanted cometary ions, entrained by electromagnetic fields associated with the plasma flow, gain much of the deceleration energy², having 40% of the total pressure behind the shock in the transverse field situation calculated by Galeev *et al.*¹⁰. When they undergo charge-exchanging reactions, such as



most of their energy and momentum is lost with the neutralized particle. This is sufficient to drive D [equation (1)] negative at low enough Mach number. When the ions are highly suprathermal, D can go negative at Mach numbers little less than unity¹⁰, as in a Venus interaction model².

An additional loss process for the energetic cometaries arises because they drift across the bulk plasma flow threaded by a magnetic field that is draped around the comet on the large scale^{11,12}. In such a field structure, fresh ions originating in the sunward coma generally diverge from the symmetry axis¹². Their mean effect is additional cooling and a loss of momentum that supplement the losses through charge exchange. The situation differs from that investigated by spacecraft at Venus or in the artificial AMPTE (Active Magnetospheric Particle Tracer Explorers) comet, where the obstacle and boundary layer scales were smaller than gyroradii of new ions. In such cases (see ref. 13), charge separation results in sheaths and streaming instabilities with intense, short-wavelength electric fields.

In the presence of strong cooling, Galeev *et al.*¹⁰ proposed a solution with re-accelerated flow and excluded any stagnation region. But that depends on the assumption of incompressibility:

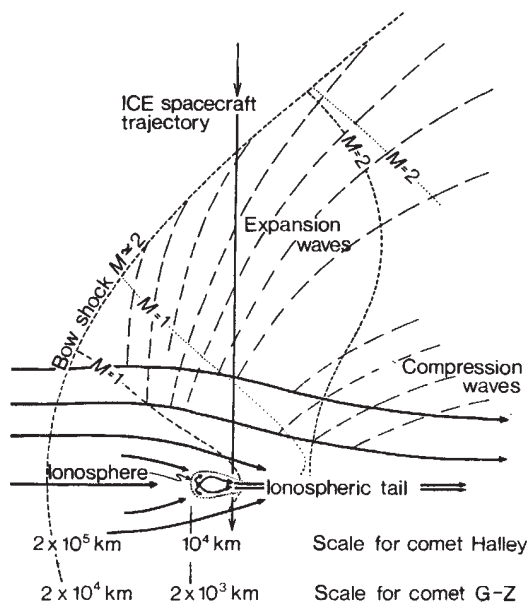


Fig. 1 Plasma flow through the coma under strong cooling. Schematic streamlines and expansion/compression wave patterns are sketched using the $M = 1$, $M = 2$ lines displaced upstream from those computed for mass-accreting flow (dotted lines).

$uA = \text{constant}$ which, combined with equation (2), requires $du = -Ddz/\rho > 0$. We choose the alternative behaviour, whereby streamtubes tend to reconverge due to low inner pressure: then $dA \approx 0$, which allows $du < 0$ [for $Ddz < 0$] continuing into the stagnating flow.

In this case, the flow pattern would be as sketched in Fig. 1. There is a small ionosphere plus tail of relatively cool plasma in the interior, with a stagnation region ahead but converging streamlines farther out. The sonic line is displaced sunwards from that computed in mass-source models⁷⁻⁹ and Mach numbers are higher behind the cooled coma, as indicated by the $M = \sqrt{(\rho u^2/\gamma p)} = 2$ line. The intermediate region of the coma acts more like a 'sink' than an ion 'source' due to its cooling power. The negative D acts to accelerate the supersonic flow². Qualitatively, we use gas-dynamic arguments^{4,14} to sketch patterns of expansion waves emitted from convex arcs of streamlines. Such expansion waves propagate out at the angle $\arcsin(1/M)$ to the flow and on reaching the flanks of the bow shock weaken it. They also bend the bow shock more sharply downstream. If the flow close to the ionosphere is primarily converging (as estimates indicate), the total weakening will be sufficient to cause complete decay of the shock's limbs, leaving Mach waves or propagating Alfvén waves. Of the two cases we discussed earlier¹⁵, the heliosphere with cooled, convergent wake is closest to the present case. The concave downstream streamlines emit compression waves, but the tailside coma provides decreasing friction ($S \uparrow 0$) like a supersonic diffuser, giving increasing Mach number in the emergent flow: the waves may, therefore, diverge as in Fig. 1, rather than converge to the classical tail shock. The innermost ionospheric tail can still, in principle, have the complex wave pattern of a supersonic jet¹⁵.

The gas-dynamic description of equation (2) needs modification to describe the magnetized flow properly. However, the differences are small when the field is weak, as is the case for energy density some 2–5% of the flow energy. Then, the magnetic field behaves primarily as though convected outside 0.1 times the shock scale¹¹. There is a magnetic contribution to the pressure with an effective γ of 2, which limits the potential compression effected by cooling. In addition, Maxwell stresses transfer some momentum from the solar plasma that is convecting the outer parts of draped field lines, to the cometary plasma that's dragging back the inner parts. The net result is extra acceleration of the sheath around the tail wake and ionosphere (Fig. 1) compared with the gas-dynamical case, but there is no qualitative difference

compared with our flow pattern.

The ICE spacecraft is targeted to fly through the wake of comet Giacobini-Zinner at $>10,000$ km downstream, that is at >0.3 times the shock stand-off scale¹⁶. It is hoped that the location of the shock flanks and the size of density and field jumps will reveal the nature of the shock and how far the solar wind "is deflected around or mingles with cometary plasma"¹⁷. We suggest, however, that interpretations will not be straightforward. The bow-shock flanks will be less widely flared and weaker than those given by the mass-source model and may be fully decayed. The main signature of the solar wind interaction will be the presence of energetic cometary 'pick-up' ions, whose places of origin will be complicated by significant drifts relative to solar protons.

The ICE spacecraft flew through the comet tail on 10 September at 8,000 km downstream. Initial results¹⁸ show an ionospheric tail and energetic ions over an extensive region but no bow shock, in agreement with our present prediction.

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Underwater noise caused by precipitation

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The characteristics of underwater noise in the ocean generated by precipitation are important to weather forecasters and oceanographers because they permit the detection and measurement of rain over the oceans by remote (buoyed or bottom-mounted) acoustic sensors. We have recently observed the characteristics of the underwater noise generated by rain, hail and snow. The spectrum of rain noise, for wind speeds below 1.5 m s^{-1} , shows a peak at 13.5 kHz with a sharp cutoff on the low-frequency side and a gradual fall-off (7 dB per octave) on the high-frequency side. Stronger winds smear the peak. Hail spectra show a peak at 3.0 kHz with a gradual (roughly 11 dB per octave) fall-off on both sides. The spectrum of snow noise is unique. Our instrumentation permitted the measurement of the drop (or stone) size distributions in the precipitation. These findings will enhance the art of remote acoustic sensing in oceanography.

Traditionally, the study of undersea acoustic noise has remained the province of the sonar designer and his co-workers because it sets limits on the detection capabilities of sonar equipment. The characteristics of such noise have been determined by many workers¹⁻⁴ and the primary causes have been attributed to wind, shipping and, in shallow water, to biological organisms.

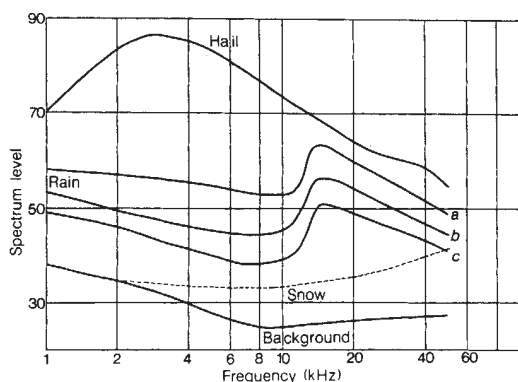


Fig. 1 Underwater noise spectra of rain (a-c), hail and snow for wind speeds $> 1.5 \text{ m s}^{-1}$. Spectrum level is expressed in dB relative to $1 \mu\text{Pa}^2 \text{ Hz}^{-1}$. Rain rates: a, 1.1 mm h^{-1} ; b, 0.29 mm h^{-1} ; c, 0.13 mm h^{-1} . Wind speeds: a, 2.7 m s^{-1} ; b, 2.7 m s^{-1} ; c, 2.23 m s^{-1} .

In recent years, 'the inverse problem' has been in vogue. Here the underwater noise becomes the signal, and measurement of this quantity in deep water appears to constitute a valid measurement of wind speed above the water surface. Measurements of wind speed and undersea noise level exhibit a high correlation, although relatively short-duration peaks in the noise records were attributed to rain by Shaw *et al.*⁵, who suggested the use of two or more observation frequencies to distinguish between wind- and rain-induced noise. Lemon *et al.*⁶ showed that undersea noise measured in three frequency bands centred at 4.3, 8.0 and 14.5 kHz on a continental shelf was readily inverted, through a simple logarithmic relationship, to wind speed within $\pm 1.5 \text{ m s}^{-1}$, for wind speeds in the range $0\text{--}12 \text{ m s}^{-1}$, and also obtained estimates, using Franz's⁷ data, of rainfall amounts which agreed within a factor of 2 with those obtained at nearby shore stations.

The undersea noise induced by precipitation has received little attention. Franz⁷ developed a theoretical expression for the spectrum of rain noise based on measurements of the underwater noise produced in a tank by sprays of droplets from a form of shower head. Heindsmann *et al.*⁸ reported some observations of undersea noise intensity resulting from rain showers in Long Island Sound, while Bom⁹ obtained spectra of underwater rain noise in the 1,200–9,600-Hz frequency band in Sarzana Lake in northern Italy. Nystuen¹⁰ has reported on some broadband measurements made in Clinton Lake.

During the winter of 1984–85, we made selected observations of underwater noise produced by carefully monitored precipitation in a roughly 4-km^2 arm at the eastern end of Cowichan Lake, South Vancouver Island, British Columbia, Canada ($48^\circ 48' 15'' \text{ N}$, $124^\circ 10' 12'' \text{ W}$). The forms of precipitation observed were rain, hail and snow, observations of the latter two forms being by chance rather than from experimental intent.

The hydrophone (an ITC 8084A having a near-spherical spatial response and a quasi-flat frequency response over the

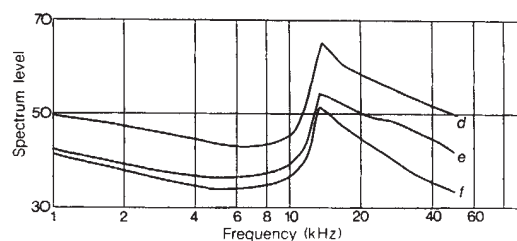


Fig. 2 Underwater noise spectra of rain for wind speeds $< 1.5 \text{ m s}^{-1}$. Spectrum level is expressed in dB relative to $1 \mu\text{Pa}^2 \text{ Hz}^{-1}$. Rain rates: d, 0.7 mm h^{-1} ; e, 0.2 mm h^{-1} ; f, 0.11 mm h^{-1} . Wind speeds: d, 0.76 m s^{-1} ; e, 0.59 m s^{-1} ; f, 0.45 m s^{-1} .

range $1\text{--}50 \text{ kHz}$) was mounted 0.7 m from the lake bottom in 35 m of water some 300 m from shore and its signal was carried by cable, after pre-amplification, to a recording facility ashore. After further amplification and improvement in the frequency response (flat $\pm 0.5 \text{ dB}$) over the frequency range, the signal was fed to an HP 3580A spectrum analyser using a 300-Hz -wide swept filter, which provided adequate resolution for the form of spectra observed and presented here. Wind speed and rain rate measurements were made ashore on a flat, bare tract of land virtually co-planar with the water surface, using conventional Government of Canada equipment and a Distromet RD69 distrometer.

Figure 1 shows underwater noise spectra due to hail, rain (a-c) and snow. The three rain noise spectra, corresponding to the rain rates given in Table 1, are typical of the bulk of the spectra for light rain which were collected. Figure 1 illustrates the characteristics of the rain spectra for wind speeds of $\sim 2.6 \text{ m s}^{-1}$ through a constant fall-off in sound level of about 7.0 dB per octave for frequencies increasing above 18 kHz , a rounded peak in the frequency interval $12\text{--}18 \text{ kHz}$ and a rapid fall-off in sound level of roughly 45 dB per octave for frequencies decreasing below 12 kHz . Below 8 kHz , these spectra show more gradual slopes of $\sim 4 \text{ dB}$ reduction in level for each octave increase in frequency.

The noise spectrum produced by hail is quite different from that of the rain noise, showing a single broad peak centred at 3 kHz , with a fall-off of $10\text{--}12 \text{ dB}$ per octave on the high- and low-frequency sides of the peak respectively. As one might expect, the noise spectrum due to snow is of low level, and is characterized by a gradual increase of the order of $3\text{--}5 \text{ dB}$ per octave as the frequency increases.

Figure 2 shows a set of rain noise spectra taken in calm conditions. In contrast to those shown in Fig. 1, these spectra exhibit a very sharp cutoff at 13.5 kHz . The fall-off on the high-frequency side of the peak is between 6.5 and 10 dB per octave, essentially the same as those of the rain noise spectra taken with wind speeds of 2.6 m s^{-1} , while on the low-frequency side the spectral slope is $55\text{--}65 \text{ dB}$ per octave. The low-frequency slope ($< 5 \text{ kHz}$) is 3 dB per octave, with the level decreasing

Table 1 Drop size distributions in terms of the number of raindrops (or hailstones) in a given diameter interval falling on 1 m^2 of the surface per s

Spectrum	Drop diameter interval (mm)															Total no. of drops ($\text{m}^{-2} \text{ s}^{-1}$)	Mean rain rate (mm h^{-1})	Kinetic energy (dB) re spectrum f
	0.3-0.4	0.4-0.5	0.5-0.6	0.6-0.7	0.7-0.8	0.8-1.0	1.0-1.2	1.2-1.4	1.4-1.6	1.6-1.8	1.8-2.1	2.1-2.4	2.4-2.7	2.7-3.0	3.0-3.3			
a	7	2	33	74	87	174	74	54	20	9	7	0	0	0	0	541	1.06	25
b	9	9	27	67	54	74	34	9	2	0	0	0	0	0	0	285	0.3	18.5
c	2	14	20	60	33	47	7	2	0	0	0	0	0	0	0	185	0.166	14.0
d	134	107	275	268	167	140	40	7	0	0	0	0	0	0	0	1,138	0.721	21.1
e	214	208	141	74	20	20	7	0	0	0	0	0	0	0	0	684	0.211	12.9
f	0	0	0	7	2	2	0	0	0	0	0	0	0	0	0	11	0.01	0
Hail	2	2	2	20	60	268	301	181	127	114	114	74	54	7	2	1,328		

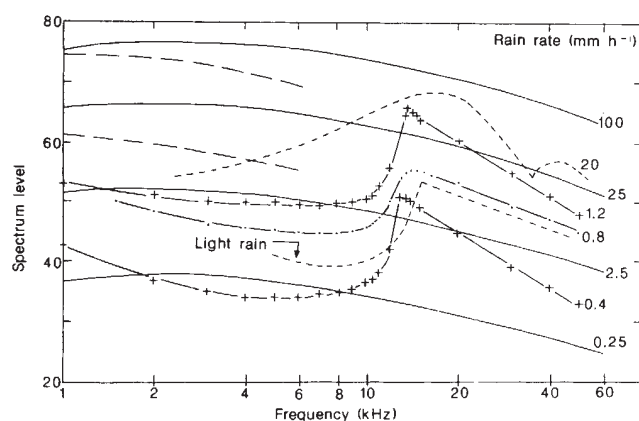


Fig. 3 Comparison of rain noise spectral samples due to Franz (—), Bom (—), Nystuen (---) and Scrimger (wind $<1.5 \text{ m s}^{-1}$ (+—+), wind $>1.5 \text{ m s}^{-1}$ (—)). Spectrum level is expressed in dB relative to $1 \mu\text{Pa}^2 \text{ Hz}^{-1}$. Numbers adjacent to curves are rain rate in mm h^{-1} .

with increasing frequency. Figure 3 compares our results with those reported by other workers.

The use of a distrometer permitted the determination of the drop size distributions in the rain producing the acoustic noise samples. These are given in Table 1, which shows the number of drops in a given drop-size interval, expressed in terms of the drop diameter, falling on 1 m^2 of the surface per s. Also given is the rain rate derived from the drop counts and the power input per m^2 at the surface due to the kinetic energy of the drops, expressed in dB, referred to the power associated with the rain noise of spectrum f . The latter was chosen as it was obtained from the lightest rainfall of the samples considered. The distrometer samples were 30 s long, so that three contiguous samples were added to correspond to a single 90-s sample obtained in near coincidence with the 100 s needed to produce the acoustic spectrum.

These data are remarkable for the abrupt fall-off in level of the rain noise spectra below 13 kHz and particularly for the sharp contrast in form between the spectra of the rain noise taken in winds of speeds $<1.5 \text{ m s}^{-1}$ compared with those taken in winds of greater speed. In the 17 spectra obtained in winds of speeds below 1.5 m s^{-1} , which might well be considered 'pure' rain noise spectra, the peak occurs at 13.5 kHz within a few hundred hertz and the cutoff on the low-frequency side of the peak is extremely sharp, dropping the spectrum level over 15 dB in a 2-kHz frequency interval. Evidently, the wind, as it increases in speed above 1.5 m s^{-1} , affects the noise generation mechanism in such a way that the 13.5-kHz peak is rounded off and extended over several kHz and the slope on the low-frequency side of the cutoff is diminished. The hail spectrum above 15 kHz is essentially the same as the rain spectra but it does not exhibit the cutoff near 13.5 kHz, and peaks at 3.0 kHz. Considering the different properties of hail and rain—the rigidity of the hail and the fluidity and surface tension of the rain—the simplicity of the hail spectrum compared with that of the rain is not surprising. Also, *a priori*, the negative slopes of the respective spectra towards high frequencies are consistent with the concept that the higher-frequency energy in the noise is generated by the smaller drops (or stones) and that these input less energy (both kinetic and, in the case of rain, potential) at the water surface.

The noise spectrum of falling snow is unique in that it increases uniformly at 3 dB per octave as frequency increases over its entire range. As the kinetic energy of the falling snow is negligible compared with hail or rain, the noise must arise from a process associated with the melting of the snow.

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Polymerization of silicate anions in solutions at low concentrations

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Adsorption of tetrahedral-forming ions, such as silicates and phosphates, is considered to be the main cause of retention of these anions on the surfaces of hydrated Al- and Fe-oxides. The importance of this retention in natural and man-caused phenomena has been the subject of many investigations. When adsorption studies are conducted, the role of the surface and the charges developed on it by the potentially determining ions tend to be emphasized, while the nature of the sorbate is rarely considered or investigated. Nevertheless, the excluded or equilibrium solution is the seminal source of information in the formulation of adsorption mechanisms or models. The ionic species present in the equilibrium solution are practically always taken for granted, that is, from data previously published. They are thought to be a simple function of solution pH, without further analysis of ionic speciation changes that occur during the sorption process. Here, we provide direct experimental evidence, using an advanced and sensitive spectroscopic technique, laser Raman spectroscopy (LRS), that aqueous equilibrium silicate solutions at low concentrations contain polymeric and/or other anionic complexes and not just monomeric species. These low concentrations are similar to those used in most previous sorption experiments on Fe- and Al-sesquioxides and on tropical acidic soils. Our findings clearly indicate that any mechanisms and models that are proposed to describe anion sorption on sesquioxides should consider that silicates, and probably some other tetrahedral-forming anions, are not present in solution solely as monomeric ionic species as most investigators have assumed^{1–11}. Irregularities in fitting experimental data to models previously reported prompted this research^{2–4,6,12}.

We determined that a spectrum of Na- or Ca-silicate could be obtained at very low Si concentrations and that the 'fingerprint' approach (Fig. 1) is applicable to the spectra obtained because they have lines distinctly placed, well separated, and of adequate intensity to determine the frequencies at which the peaks or bands occur. The published Raman spectra of Na silicates in solution were determined from Si solutions of concentrations 200–1,000 times larger than the solutions used in this study^{13–16}. No laser Raman (LR) spectra of Ca-silicates in solution have been published.

The spectrum of a Na-silicate solution at a Si concentration of $75 \mu\text{g cm}^{-3}$ is presented in Fig. 1. Numerous precautions were taken to exclude CO_2 from the alkaline solutions. Air-tight plastic bottles were used to store the silicate solution and all of the samples were freshly prepared just before the experiments with degassed water. The solution pHs were measured with a combination electrode immersed in a sealed plastic container to restrict any CO_2 contamination. Unless otherwise stated, all the solutions examined were adjusted to pH 10 since this pH is close to the reported pK of monosilicic acid, that is 9.5 (refs

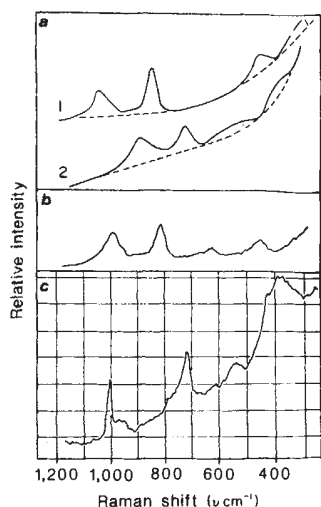


Fig. 1 Comparison of spectra of Na_2SiO_3 solutions reported by different authors. *a*, Raman spectrum as reported by Fortnum and Edwards¹³. 1, NaH_2PO_4 , 4 M solution (used as silicate analogue); 2, Na_2SiO_3 , 2.5 M. *b*, Laser Raman spectrum of Na_2SiO_3 1.5 M as reported by Freund^{14,15}. *c*, Laser Raman spectrum of Na_2SiO_3 (multipass device) ($\text{Si} = 75 \mu\text{g per g} \approx 0.003 \text{ M}$).

7-9). This pK_a is a crucial component of the conclusions of many previous investigations. As a basis for comparison, Raman spectra of 1.5 and 2.5 M silicate solutions previously reported¹³⁻¹⁵ are also shown. The remarkable intensity of the Raman lines for the low Si concentration solution is attributed to the enhancement of the signal due to the multipass device used in the studies. A very satisfactory spectrum may also be obtained with Si concentrations as low as $30 \mu\text{g cm}^{-3}$. More details of the methodology and the analogue structures suitable for these analyses will be published elsewhere.

Consideration of the position and number of the Raman shifts in the $400\text{--}500 \text{ cm}^{-1}$, 600 cm^{-1} , 785 cm^{-1} , and at the ≈ 900 and $1,050 \text{ cm}^{-1}$ regions agree fairly well. The slight displacement in the 900 and $1,000 \text{ cm}^{-1}$ regions may be due to pH differences. Solutions that are 1.5 or 2.5 M obviously have much higher pHs (> 12) than the $25\text{--}75 \mu\text{g cm}^{-3}$ working range of our Si solutions.

Calcium as well as Na salts are frequently supporting electrolytes in sorption studies, and soluble silicates occur at low concentrations in the soil solution, and in numerous other aqueous environments. When we examined solutions of low Si concentrations, 'anomalies' led to the suspicion that silicate species other than monomers were present. The indications of silicate polymerization or the formation of complexes other than monomeric silicates or silicic acid are reflected in the following LR spectral characteristics: (1) the band at $1,070\text{--}1,090 \text{ cm}^{-1}$ is considered to indicate the presence of a dimer (Si-O-Si band)^{13-15,17} or Si polymers^{13-16,17-20}; (2) changes in the band features, that is, splitting and/or broadening of peaks, shifts of the band frequencies to higher values, and changes of intensity (band height). The lines at $\approx 780 \text{ cm}^{-1}$ are especially sensitive to these changes^{14,15,17-19,21,22}.

The increase in polymerization of the silicate solution is reflected qualitatively by an increase in the intensity of the Raman lines at 780 and $1,070 \text{ cm}^{-1}$ (Fig. 2) in spectrum *b* compared with spectrum *a*. With increasing Si concentration, polymerization of Si rapidly occurs as shown by the development of the peak at $1,070 \text{ cm}^{-1}$ (refs 15, 16, 19-22).

Using times and ionic environments comparable to most other previous sorption studies^{1-6,12}, we determined that a silicate solution of low concentration is not only in monomeric form. Fresh monosilicic acid solutions ($75 \mu\text{g cm}^{-3}$) were prepared using the H-resin method⁸ and brought to pH 10 with NaOH and examined immediately by LRS (Fig. 3). Spectra were run at 15-min intervals and when the solution 'aged' at 3 h, distinct changes in the spectrum as appears in Fig. 3 inset *b* were clearly noticed.

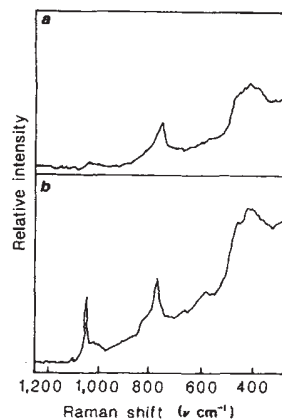


Fig. 2 Laser Raman spectra of aqueous solutions of Na_2SiO_3 with varying concentration. The increase in polymerization in graph *B* is reflected by an increment in the band $\approx 1,070 \text{ cm}^{-1}$, which corresponds to a silicate dimer (Si-O-Si band) or higher polymer. *a*, Na_2SiO_3 solution at $75 \mu\text{g cm}^{-3}$; *b*, Na_2SiO_3 solution at $100 \mu\text{g cm}^{-3}$.

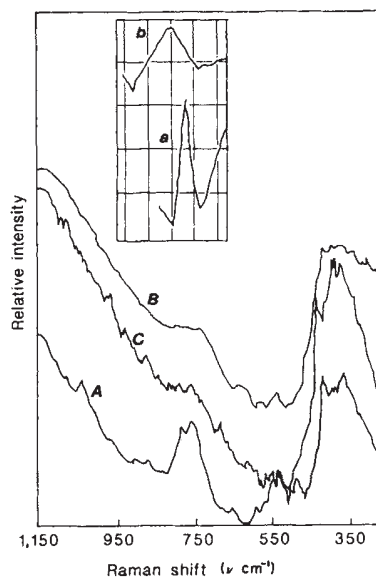


Fig. 3 Laser Raman spectra of Si-solutions at a Si concentration of $75 \mu\text{g per g}$. Ca(OH)_2 or Na(OH) were added to reach a pH = 10. This pH was selected because between pH 8.5 and 10.5 'maximum adsorption' or 'adsorption envelopes' are reported to occur. *A*, A freshly prepared solution of monosilicic acid and Ca(OH)_2 . *B*, Solution *A* examined after 'ageing' for 3 h after exposure to the laser beam. *C*, Solution *A* examined after ageing for 3 h with no previous exposure to the laser beam. No differences between *B* and *C* were detected, therefore the Si-solution was not polymerized by photocatalysis. Inset: *a*, A freshly prepared solution of monosilicic acid and NaOH. *b*, Solution *a* after ageing for 3 h. The $\approx 782 \text{ cm}^{-1}$ peak broadens, splits and shifts frequency as a sign of polymerization. The labelling of the axes are as in *A*.

The 782 cm^{-1} frequency observed (Fig. 3, insets *a* and *b*) was taken as a reference and it was shown from experimental evidence^{17,22} that the ≈ 780 peak is perturbed when Si polymerizes. This is evident because inspection of the spectra revealed three very distinct alterations of the aged solution in comparison with that at 'zero-time', (Fig. 3, insets *a* and *b*): (1) the band width broadened to twice the value at 'zero-time'; (2) the intensity (band height) decreased nearly 50%; (3) the frequency was displaced from 782 to 803 cm^{-1} . Effects of photocatalysis, due to the laser beam, on the rate of polymerization were ruled out by additional experiments in which Ca was the complementary cation (Fig. 3, *A*, *B*, *C*). Although not shown, a sample of $30 \mu\text{g cm}^{-3}$ Si was examined in similar conditions and even at

this concentration, variations of the 782 cm^{-1} peak occurred 3 h after sample preparation.

The present results are simply a predictable extension of proven facts which explain polymerization, anion bridging and/or gelation of inorganic polymers from solutions at higher concentration than in our studies^{8,9,21,23-28}. They indicate that it is difficult to define a boundary Si concentration, high or low, where silicate monomers are the sole species, which has been the repeated assumption in many previous investigations. Our results confirm that the formation of at least a silicate dimer, at pH 10, predicted from theoretical and experimental reported data^{23,28} occurs at any silicate concentration (in fact, 35% as silicate-dimer, Iler, unpublished data). Even at low concentra-

tions and after a short period of time, a substantial part of the silicate solution is non-monomeric. Moreover, the widely accepted concept that maximum adsorption of silicate on sesquioxides occurs at the $pK_a = \text{pH}$ of monosilicic acid^{1-6,12} without consideration that polymeric species exist has led to incorrect explanations and non-fitted models for those 'adsorption' systems. At $\text{pH} = pK_a$ the conditions are optimal for polymerization to occur^{25,26}. Indeed, our statements do not proscribe sorption of silicates on various sesquioxides in nature and in the laboratory^{1-6,12,22}. Work is in preparation which explains polymerization mechanisms at low concentration, and the possible configurations of dimers and polymeric species, based on evidence from spectroscopy and complementary techniques.

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Laboratory models for aromatization and isomerization of hydrocarbons in sedimentary basins

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With increasing depth in the Earth's crust, changes take place in the sedimentary distributions of aromatic steroid hydrocarbons and of stereoisomers of certain types of alkanes^{1,2}. The changes occur before and during the early stages of hydrocarbon generation and have been interpreted as resulting from aromatization and configurational isomerization reactions respectively³. We have now brought about an aromatization reaction and an isomerization reaction in the laboratory under free radical conditions, sulphur being chosen as a convenient source of radicals, and have extracted rate constants from the absolute concentration-time functions of the organic substrates. The activation energies and pre-exponential factors were then deduced from the temperature dependence of the rate coefficients. The values of these activation parameters show that, in the laboratory, the aromatization is more temperature dependent than the isomerization reaction, as suggested previously from the distributions of steroidal compounds in sedimentary basins³⁻⁵.

The relative extents to which the proposed reactions occur for steroid hydrocarbons in the sedimentary column have been suggested as being indicative of the thermal history of sediments³. These data, in conjunction with a geophysical model describing sedimentary basin formation, have been used to derive both activation energies and pre-exponential factors governing the rates of the reactions⁴⁻⁶. However, such studies have used kinetic schemes based on the following assumptions: (1) the reactions obey unimolecular first-order rate laws, and (2) the total concentration of reactant plus product remains constant throughout each reaction. In addition, rate coefficients have been derived from the relative abundances of presumed products to reactants, as measured from peak areas in mass chromatograms, rather than from absolute concentrations. The activation energy of a reaction is dependent upon its mechanism⁷; hence, if laboratory-derived values are ever to be extrapo-

lated to the sedimentary column, the same mechanism must be followed in both cases. Previous authors⁸⁻¹² have proposed the importance of free radical mechanisms in petroleum generation and associated processes. Significant concentrations of free radicals have also been measured, even at room temperature, in many kerogens¹³.

In the present study, intimate mixtures of the organic substrate, powdered sediment and elemental sulphur were heated at temperatures where high concentrations of sulphenyl and polysulphenyl radicals are observed¹⁴. Typically, the mixture in Pyrex glass tubes sealed *in vacuo* (after three N₂ purges) was heated in an oven and the temperature monitored using a chromel-alumel thermocouple. The substrate for aromatization was an isomeric mixture of ring-C monoaromatic hydrocarbons (compounds I, Fig. 1)¹⁵ and the matrix was a solvent-extracted carbonate sediment (Cretaceous, Abu Dhabi), with 0.08% w/w of elemental sulphur added. For the isomerization, one stereoisomer [(6R,10S) = *meso*-pristane] of 2,6,10,14-tetramethyl pentadecane (IIIa, Fig. 1) was used, with a solvent-extracted shale as matrix (Semécourt shale, Lower Toarcian,

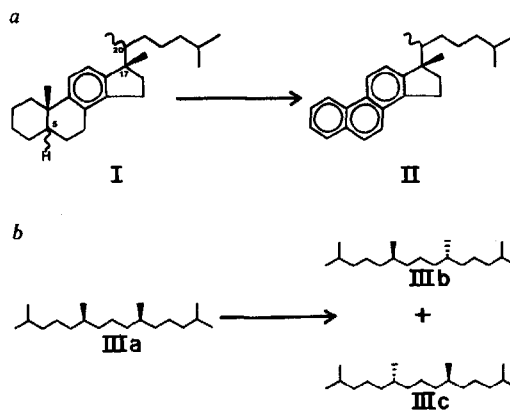


Fig. 1 a, Aromatization of the $5\alpha(\text{H})$ and $5\beta(\text{H})$ isomers of (20R)- and (20S)-17 β -methyl-18-norcholesta-8,11,13-triene (I) to (20R)- and (20S)-17 β -methyl-18,19-dinorcholesta-1,3,5(10), 6,8,11,13-heptaene (II) (numbering based on steroid skeleton). b, Isomerization of (6R,10S)-pristane (IIIa) to (6R,10R)- plus (6S,10S)-pristane (IIIb and IIIc respectively).

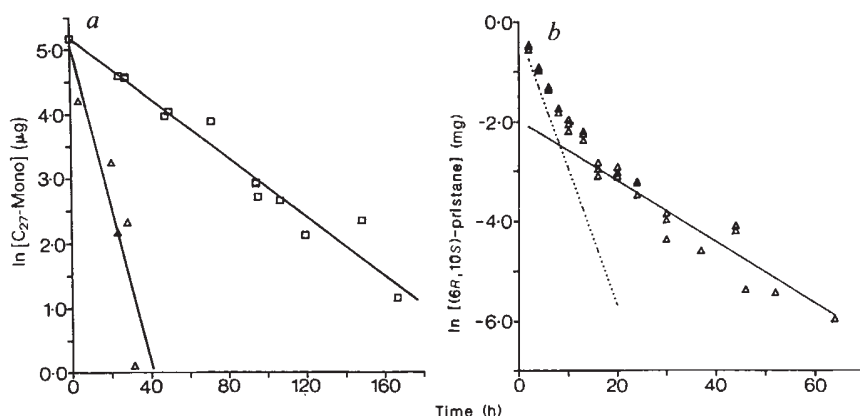


Fig. 2 *a*, A plot of $\ln[I](t)$ against heating time (h) where $[I](t)$ is the concentration of C_{27} ring-C monoaromatic steroid hydrocarbons. $\square$, 147.5 °C (420.5 K); $\triangle$, 165 °C (438 K). *b*, Plot of $\ln[IIIa](t)$ against heating time (h) where $[IIIa](t)$ is the concentration of (6*R*,10*S*)-pristane at 250.2 °C (523.4 K).

Paris Basin) plus 10% w/w of elemental sulphur. After dichloromethane extraction, quantitative product analysis was achieved by the internal standards method, using capillary gas chromatography on OV-1 (or CP-Sil 5) and DEGS/PEGS (3/1) stationary phases¹⁵.

With increasing burial depth in sediments, an increase occurs in the relative abundance of triaromatic (base peak at m/z 231 in their mass spectra; for example compounds II) to monoaromatic steroid hydrocarbons (base peak m/z 253; for example I)^{2,16}. A similar change in the aromatic steroid distribution has been observed by heating a shale in the laboratory¹⁷. Furthermore, heating experiments similar to those described here have suggested the following reaction scheme¹⁵:



where k'_A = aromatization rate constant.

If the aromatization obeys a first-order rate law, then the predicted concentration-time function $[I](t)$ for the monoaromatic steroid is:

$$[I](t) = [I](t=0) \exp(-k'_A t) \quad (2)$$

Thus, if equation (2) is correct, a plot of $\ln[I](t)$ versus time will yield a straight line with slope k'_A and intercept $\ln[I](t=0)$. In the present study, there was an exponential decrease in monoaromatic concentration with increasing time and a concomitant increase in triaromatic concentration in the early time regime. Substitution in equation (2) of experimental data from two temperatures (147.5 ± 1.5 °C and 165.0 ± 1.0 °C) are plotted in Fig. 2*a*. Linear regression analysis gave close to straight-line fits with calculated intercepts 5.20 (147.5 °C) and 5.13 (165.0 °C). These match satisfactorily the actual value (5.17) from the initial concentration of monoaromatic steroids. The rate constants extracted from the slopes have values of 0.0228 h^{-1} (147.5 °C) and 0.1246 h^{-1} (165.0 °C). Straight-line fits were also obtained at two other temperatures (Table 1).

The temperature dependence of a rate constant may be used to deduce the activation parameters of a reaction by use of the Arrhenius expression:

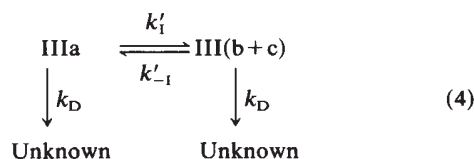
$$k = A \exp(-\Delta E_a/RT) \quad (3)$$

where R is the gas constant and T the absolute temperature.

The Arrhenius plot for k'_A , using data from Table 1, is shown in Fig. 3*a*. The slope yields an activation energy, ΔE_a , of 145 kJ mol⁻¹ and a pre-exponential factor A , extracted from the intercept at $1/T = 0$ of $\sim 6.7 \times 10^{12} \text{ s}^{-1}$.

In immature sediments, pristane occurs predominantly as the (6*R*,10*S*)-diastereoisomer (IIIa); with increasing thermal stress, a 1:1 mixture of the (6*R*,10*S*) to the (6*R*,10*R*) plus (6*S*,10*S*) isomers is eventually formed (IIIa to IIIb plus IIIc)^{1,18}. The same effect has been observed when a shale is heated in the laboratory¹⁷. In the present work no isomerization was observed when the pure (6*R*,10*S*)-substrate was heated alone in a medium of liquid sulphur. When heated on the shale matrix with sulphur, absolute quantification reveals that, in parallel with isomeriz-

ation at C-6 and C-10, there is a degradative process depleting the total amount of pristane, as proposed previously¹⁵. These observations are consistent with the following reaction scheme:



The rate constants for the two side reactions are assumed to be equal because of the diastereomeric relationship of the product to the precursor in this acyclic alkane. Additionally, rate constants for the forward and reverse isomerization are assumed to be equal because a 1:1 equilibrium mixture of precursor and product is formed. Analysis of scheme (4), with the assumption of first-order kinetics, predicts that the concentration-time function for (6*R*,10*S*)-pristane should behave as follows:

$$[IIIa](t) = a_1 \exp(-\lambda_1 t) + a_2 \exp(-\lambda_2 t) \quad (5)$$

where

$$\lambda_{1,2} = k'_I + k_D \pm k'_I \quad (6)$$

The $\lambda_{1,2}$ are the roots of the auxiliary equation:

$$\lambda^2 - 2(k'_I + k_D)\lambda + k_D(2k'_I + k_D) = 0 \quad (7)$$

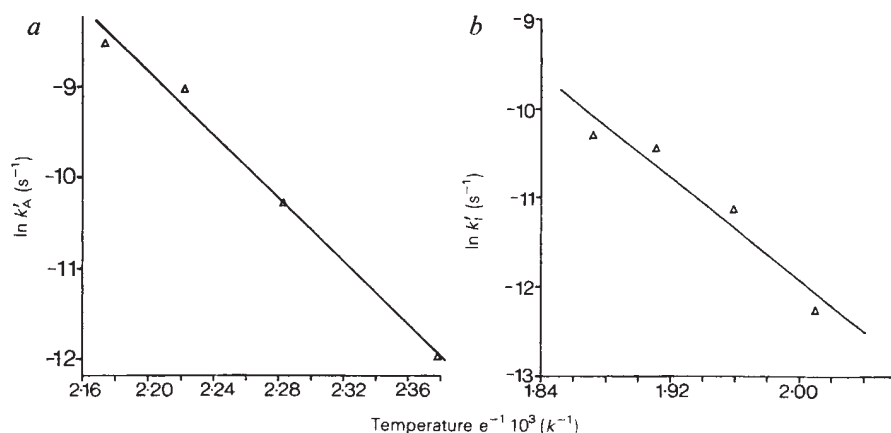
Equations (5) to (7) predict that the reactant concentration-time function should consist of two decreasing exponentials. Figure 2*b* shows that the experimental data fit this prediction. The solid line from linear regression analysis of points corresponding to $t > 21$ h, represents the longer-lived component ($\lambda_2 = k_D$). When this line is extrapolated to $t = 0$ and subtracted from the data points at early times ($t < 21$ h), a regression line (dashed) through the resulting residuals yields the short-lived component which has the time constant $\lambda_1 = 2k'_I + k_D$. Thus, k'_I at 250.2 °C is 0.1063 h^{-1} (Fig. 2*b*). Results for this and three other tem-

Table 1 Aromatization and isomerization rate constants as a function of temperature

Aromatization	
Temperature (K)*	$k'_A(\text{h}^{-1})^\dagger$
420.5 ± 1.5	0.0228 ± 0.0015
438.0 ± 1.0	0.1246 ± 0.0270
450.0 ± 1.0	0.4418 ± 0.0186
460.0 ± 1.0	0.7206 ± 0.0575
Isomerization	
	$k'_I(\text{h}^{-1})^\dagger$
497.4 ± 1.5	0.0173 ± 0.0015
510.4 ± 1.3	0.0534 ± 0.0079
523.4 ± 2.3	0.1063 ± 0.0080
534.2 ± 1.9	0.1230 ± 0.0540

* ± Maximum observed range.

† Error = ±1 s.d.

Fig. 3 Arrhenius plots for: a, k'_A and b, k'_I .

peratures are collated in Table 1. Using expression (3) and the data in Table 1, the activation parameters were extracted from the Arrhenius plot (Fig. 3b); ΔE_a has a value of 120 kJ mol^{-1} and A is $\sim 2.1 \times 10^7 \text{ s}^{-1}$.

The laboratory heating experiments show that a steroid aromatization reaction to give the m/z 231 triaromatics and a configurational isomerization reaction can be brought about in the laboratory under free radical conditions. Although first-order kinetics appear to be followed in each case, it is likely that pseudo-first-order rate constants have been measured since there is evidence that the overall rates of reaction are dependent on sulphur concentration¹⁵.

It is difficult to know at present whether these preliminary laboratory experiments, using single compound types, represent an appropriate model for the increase in extent of aromatization of aromatic steroid hydrocarbons and in the number of isomers in certain types of alkanes observed in sediments with increasing thermal stress. On the other hand, if the aromatization and isomerization reactions do indeed occur in sediments, and if free radical mechanisms operate, the laboratory ΔE_a values may be applicable to the sediment reactions. It must be recognized, however, that the apparent A factors, measured in the laboratory, are unlikely to be applicable to sediments; they are probably enhanced in the laboratory as a result of the increased concentration of radical initiator¹⁹. Despite these complexities and the fact that the laboratory ΔE_a values differ from those derived in a semi-empirical manner, using the geophysical model⁴⁻⁶, it is noteworthy that the Arrhenius plots (Fig. 3) show that the aromatization is the more temperature-dependent reaction, that is, it has larger ΔE_a (145 kJ mol^{-1}) and A ($6.7 \times 10^{12} \text{ s}^{-1}$) values than the isomerization reaction (120 kJ mol^{-1} and $2.1 \times 10^7 \text{ s}^{-1}$ respectively). Similar behaviour is implied by the relative extents of aromatization of aromatic steroid hydrocarbons and of isomerization in the side chain of steroid alkanes in that aromatization is accelerated relative to isomerization in sediments with more rapid heating rates³⁻⁵.

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Palaeoclimatic and tectonic implications of Neogene microflora from the Northwestern Ethiopian highlands

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Climatic changes in East Africa have been well documented although the record is far from complete. Palaeoclimatic data for both the Holocene and Pleistocene¹⁻⁷ and the Pliocene¹⁻⁸ are available through fossil pollen studies. However, these data extend in a continuous manner only as far back as 3.7 Myr BP (ref. 11) largely because of the emphasis on studies related to human origins. Fragmentary data from macrofossils also exist for older periods from Eastern¹⁴⁻²⁰, Central and Southern Africa^{21,22} although the scarcity of material and the lack of isotopic dates makes it difficult to define continuity in the patterns of climatic fluctuations in East Africa. The late Miocene pollen/spore flora described here represents the first data from East Africa for this time period and provides new information on the pre-Pliocene flora and climate of the continent. The pollen flora comes from a post-8-Myr lacustrine deposit in the heart of the Northwestern Ethiopian Plateau (12° 35' N, 37° 06' E) and contains 'exotic' taxa that are now extinct. The pollen diagram is characterized by abundant wet lowland rainforest taxa and pteridophytes, a very weak representation of grasses and the total absence of conifers, indicating warm and humid climates towards the close of the Miocene. Post-depositional uplift of the Chilga area (~1,000 m) is also implied from the fossil pollen/spore assemblage.

The Chilga fluviolacustrine, lignite-bearing sedimentary sequence occurs within a graben, 60 km south-west of Gondar

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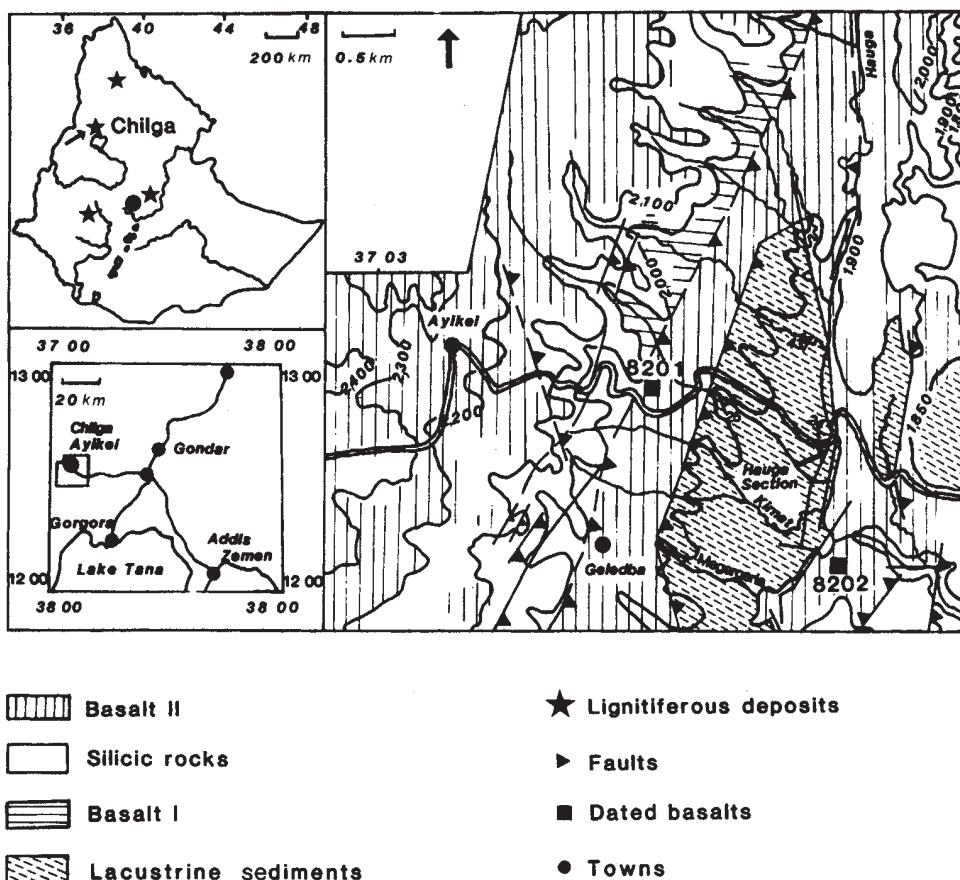


Fig. 1 Location map of Chilga and the sections studied (geological map after Tezera and Heeman²⁶).

and ~100 km north-west of Lake Tana, at altitudes between 1,900 and 2,000 m (Fig. 1). The graben is part of a large defunct Eocene rift, which ceased activity 25–20 Myr ago as the transverse-trending Afar proto-rift began to form²³. The sediments occur in one of the small north-south (NS) grabens [13 km NS by 2 km EW (east-west)] within the major Tana graben, formed by the NNE–SSW fault line running parallel to the Ethiopian Rift system. The Chilga graben is bounded on the west by highlands which rise abruptly to higher than 2,300 m. The formation of these grabens is attributed to renewed faulting activity associated with late Tertiary volcanism²⁴.

The Chilga sedimentary sequence is one of the ubiquitous continental sedimentary intercalations within the Oligo-Miocene Trap Series on the Plateau. The sequence directly overlies a basalt layer (Basalt II) which forms the graben floor. This basalt also covers the top of the westward-lying highlands and consists of numerous lava flows from separate eruptive episodes^{23,26}. It is represented by stratified, fine-grained olivine basalt (KY8201) on the highlands and is recognized along the fault line in the Chilga basin by its amygdaloidal leucite pockets and calcite veins (KY8202). Potassium–argon whole-rock analysis of two rock samples from Basalt II gave ages of 9.8 ± 0.5 and 8 ± 1.2 Myr respectively (collaboration with I. Villa, University of Pisa). The sedimentary sequence (34 m thick) commences with medium-grained basal conglomerates which fine upward to coarse-grained sandstones. It then abruptly changes to black carbonaceous mudstones which are intercalated with very fine lignite seams and thick silt layers. In the lower part, silts, clayey silts and clays dominate the section, whereas thick and consolidated lignite beds frequently occur between +15 and +30 m. A 3-m-thick water-lain volcanic ash layer crowns the sedimentary sequence.

The climate in Ethiopia is controlled by macro-scale pressure changes and monsoon flows²⁷, with elevation and topography contributing variation on the Plateau²⁸. The Northwestern Plateau is under a regime of summer rain with a mean annual rainfall of 1,000–1,400 mm distributed in one single season with

a dry period of 5–7 months²⁹. A local maximum of 1,500–1,800 mm on the mountain east of Lake Tana and 950–1,500 mm around the lake is observed¹⁸. The warmest season (25 °C) is March–May; the mean temperature on the Plateau is 5 °C cooler.

The vegetation of the Northwestern Plateau belongs to the Afromontane archipelago-like regional centre of endemism³⁰. Further south, an island of undifferentiated woodland vegetation dominated by Combretaceae associated with *Balanites aegyptica*, *Piliostigma thonningii* and *Gardenia ternifolia* form a halo around Lake Tana. Remains of evergreen montane forest with *Olea africana*, *Podocarpus gracilior*, *Juniperus procera* and *Sapotaceae* occur above 2,000 m on the well-studied east side of the Lake Tana region^{32,32}. Stands of natural vegetation have now disappeared under the pressure of human settlement³³.

Forty-six complete pollen spectra were obtained from a total of 96 studied samples collected from two outcrop exposures within the Chilga basin. Vitritine-rich lignites and coarse detrital sediments preserved no fossils, whereas intertinite-rich lignites, carbonaceous mudstones, clays and silts yielded rich pollen and spore assemblages. Identification of fossil pollen grains was achieved with the help of a reference collection of 6,000 modern species of Ethiopian and East African flora^{34,35} and the available literature on pollen morphology^{36,37}.

We recorded 121 identified and 2 unidentified pollen taxa, with only 0.5% of unknowns on the total pollen/spore count. The Chilga fossil pollen assemblage is unique in Africa in its floral composition and diversity. This fossil flora was extensively compared with post-Eocene macrofossils from the north-west, central and south-west parts of the Ethiopian plateau^{15,18,19}, Kenya¹⁴ and Uganda²⁰, with palynoflora from central, south-east and north-east Ethiopia^{38,39}, Burundi²¹ and South Africa²²; with the modern Ethiopian flora^{35,40} and with the neighbouring East African flora⁴¹. The comparisons show three striking features in the composition of the post-8-Myr flora from Chilga, namely, the presence of 'exotic' taxa, the absence of *Podocarpus* and *Juniperus*, and the abundance of pteridophytes.

The fossil flora contains taxa that are foreign to the modern,

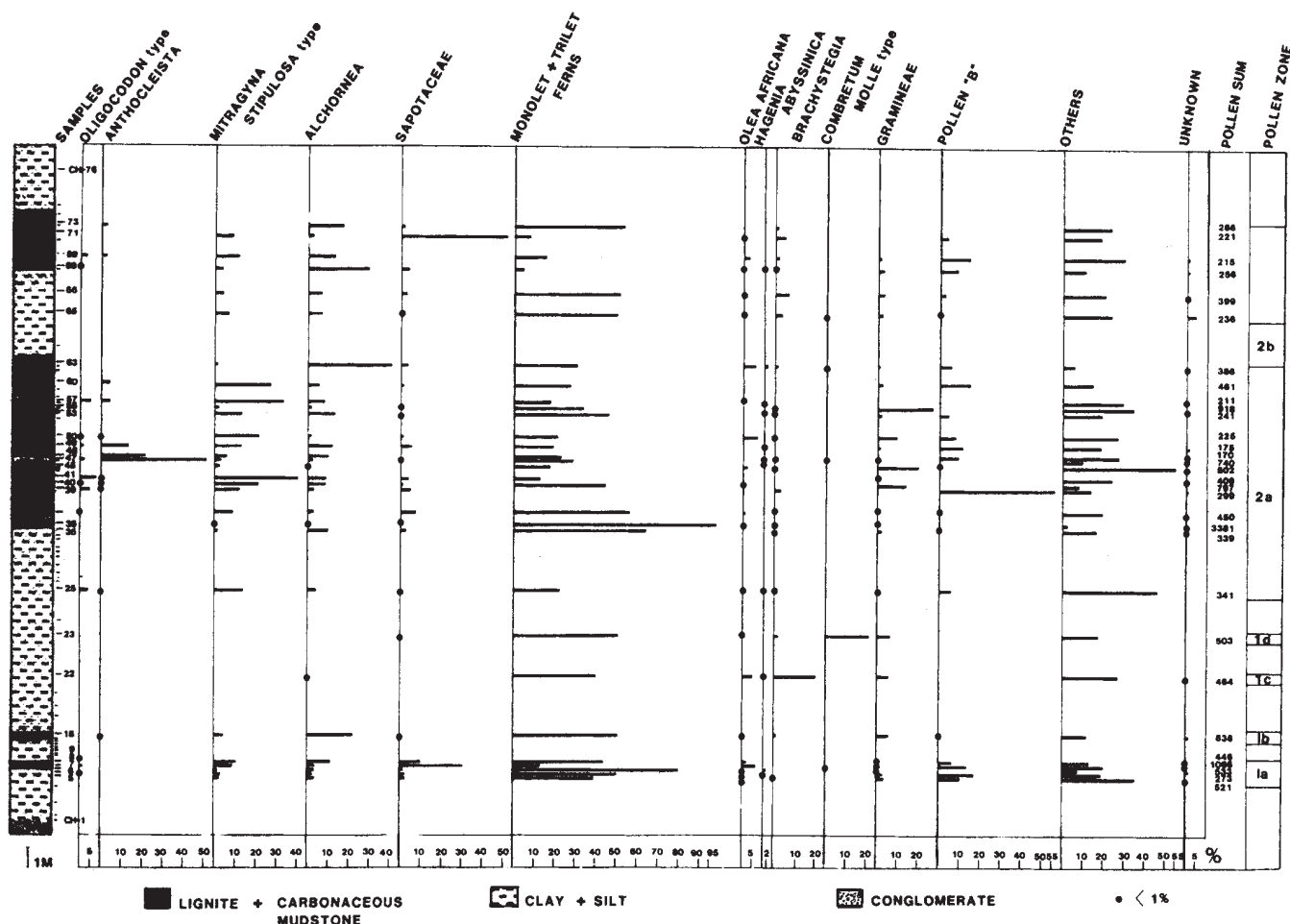


Fig. 2 Pollen diagram of the Chilga lacustrine deposit; relative frequencies calculated with respect to the total pollen/spore count.

Pleistocene and Pliocene pollen flora from East Africa³⁹. These include *Afrocrania volkensii*, *Brachystegia*, *Isobertlinia* type, *Iodes* type, *Holoptelea grandis* type, *Oligocodon* type and *Rauvolfia*. Although *Afrocrania* does not exist in the present Ethiopian flora⁴⁰, it is a major element of wet montane vegetations up to 3,200 m and down to lake water edges in moist montane forests in East Africa. The nearest *Brachystegia* and *Isobertlinia* vegetations are found today >1,000 km south of Chilga, in northern Tanzania, with a southward arm extending into Zimbabwe⁴¹. *Oligocodon*, a climbing shrub in tropical West Africa, has so far been recorded only from the wet rainforests of Nigeria and Cameroun³⁷. On the other hand, a taxon with pollen type indistinguishable from *Oligocodon*, *Gardenia imperialis*, grows along stream, lake and swamp margins in Uganda and Congo. *Holoptelea* is a widespread species in the Guineo-Congolian phytogeographical region as an important component of semi-evergreen lowland rainforests³⁰. The pollen of *Rauvolfia vomitoria* recorded from the rainforests of West Africa³⁷ has similar pollen types: *Rauvolfia caffra* and *Rauvolfia manni*, species which occur in montane forest vegetation in Burundi and Tanzania. The presence of these 'exotic' taxa in association with the abundant lowland taxa such as *Anthocleista*, *Mitragyna* and *Alchornea* strongly suggests a closed forest environment that is far more humid than present-day forests in the highlands of Ethiopia.

Podocarpus gracilior is a species that is a widespread component of upland forests throughout Ethiopia and East Africa. *Podocarpus* is found in East Africa in forests where the mean annual rainfall is within the range 800–1,600 mm at altitudes between 1,800 and 3,000 m. On the Western Plateau, it occurs between 1,500 and 2,200 m in nearly pure stands. *Juniperus procera*, the other associated conifer, is abundant in dry montane forests, at altitudes above 1,700 m and is also a major component

of the forest zone between 2,900 and 3,000 m altitude. The absence of a single pollen grain of either of these two conifers in a total pollen/spore count of 24,452, specially considering the excellent export ability of *Podocarpus*³, is solid evidence for the absence, on the regional scale, of present-day type upland forests on the plateau during the period of deposition. The absence of conifers is also one factor which physiognomically differentiates Guineo-Congolian rainforests from Afromontane rainforests³⁰.

More than seven different types of spores were observed in the Chilga pollen/spore flora. Except in the Upper Neogene palynoflora from Burundi²¹, which has an even more important spore flora, the Plio-Pleistocene palynoflora from East Africa does not show such diversified and abundant ferns as the Chilga pollen/spore assemblage. Elevated frequencies of pteridophytes denote an increase in rainfall and runoff⁴², thus suggesting that the Chilga fossil flora may represent the moist and shady conditions of a closed, wet forest.

Percentage frequencies of the pollen and spore assemblage are shown in Fig. 2 (detailed manuscript in preparation). This pollen diagram is condensed from the larger and more complete Hauga section (31 spectra). It comprises only taxa characterized by their high pollen concentration, consistent presence and/or generic significance. Three important features distinguish Chilga from other East African Tertiary sites.

(1) Dominance of arboreal taxa: the arboreal taxa make up >50% of most spectra (>70% if pteridophytes are excluded), a distribution pertaining to the structure of closed forests. No pollen diagram from the East African Plio-Pleistocene shows such high arboreal frequencies.

(2) Weak representation of grasses: most pollen diagrams from East Africa are characterized by high frequencies of *Gramineae* which often account for >50% of the total pollen count^{8,11,13},

indicating widespread savanna and open woodland vegetation in the Plio-Pleistocene. In the diagram from Ghilga, the Gramineae frequencies, where present, are low, 0–27% (maximum in the moist pollen zone IIa), and the frequencies of the sedge (mostly absent) do not reach 15% (not included in this diagram). This also suggests a closed forest environment. (3) Instability of the palaeovegetation: six distinct development phases are recorded in the palaeovegetation during the depositional period. The earliest *Mitragyna* swamp forest developed extensively with *Alchornea* (Ib) at the expense of a montane-like vegetation (Ia). Through an unrecorded transition, it gave way to a deciduous *Brachystegia* forest (Ic) which further developed into a drier phase of *Combretum*–*Lepidagathis* woodland (Id). This vegetation was then completely replaced by an *Anthocleista*–*Mitragyna*–*Oligocodon* association of a fringing swamp and wet lowland forest (IIa). Towards the end of the period of deposition, a moist secondary forest with an *Araliaceae*–*Alchornea*–*Sapotaceae* association (IIb) became dominant, with *Sapotaceae* comprising >55% of the assemblage in the uppermost spectrum. These frequent changes from a closed swamp forest to semi-deciduous forest reflect a decrease in the humidity on the plateau.

The rate of uplift for the high plateaus of Ethiopia is thought to have increased exponentially since 100 Myr ago, from <0.05 mm yr⁻¹ to 1 mm yr⁻¹ at present⁴³, bringing the Jurassic marine limestone to its present altitude of 2,500 m (ref. 44). Epeirogenic uplift in the Cenozoic occurred at ~0.1 mm yr⁻¹ (ref. 45). If we assume a uniform uplift rate of 0.1 mm yr⁻¹, the Chilga lake beds may have risen 800 m during the past 8 Myr, which tentatively places the Chilga region at an altitude of around 1,000 m during deposition. This is also supported by the presence or absence of certain 'elevation-indicator' plant taxa in the palaeofloral assemblage. *Podocarpus* has previously been used to indicate palaeoaltitudes and to infer uplift on the Western Plateau⁴⁶. The absence of both *Podocarpus* and *Juniperus* from the Chilga palaeoflora, the modern presence of these conifers in the highlands and the location of the Chilga lacustrine deposit at an elevation of around 1,900 m suggest initial depositional altitudes of around 1,000 m for the Chilga area. Active episodes of regional tectonic and volcanic activity are also recorded from the distribution of clay minerals (<2 µm) in the sedimentary sequence which shows an abrupt increase of illite and smectite towards the top of the lake beds.

One little known but important aspect of the late Cenozoic vegetation history of Africa is the extension of tropical rainforests and the timing of the establishment of the savanna. Savanna as well as lowland rainforests and montane vegetation are thought to have occupied tropical Africa since the Miocene⁴⁶. The contraction and later disappearance of the eastern Tethys Sea during the Miocene initiated the fragmentation of the closed forest. Faunal evidence indicates a shift from lowland to woodland vegetation in tropical Africa by the mid-Miocene⁴⁷. The woodland vegetation was further fragmented by the occurrence of a widespread savanna-mosaic during the late Miocene/early Pliocene⁴⁸. Significant contraction of the tropical lowland rainforests and establishment of the savanna appears to fall within the mid-Miocene. On the Western Ethiopian Plateau, macrofossils of montane forest taxa have been recorded from several sites^{16,17}. However, no fossil record documenting lowland rainforest is available, although there exists an isolated patch of lowland rainforest³⁰ about 400 km south of Chilga. The present study, however, provides evidence for the presence of a closed forest with strong Guineo-Congolian affinities as late as 8 Myr BP as far north as 12° N on the Northwestern Ethiopian Plateau. Furthermore, the lack of datable material at the top of the sedimentary sequence allows one to speculate on a link between the late Miocene global climatic deterioration and the disappearance of the humid post-8-Myr Chilga flora. The flora may either represent the humid climate and vegetation of the Plateau before the terminal Miocene salinity crisis at 6.5–5.5 Myr BP or a relict vegetation which survived the Mediterranean aridity. Whatever the case may be, a change towards a cooler

and more arid climate in the circum-Mediterranean region that has been related to global Messinian events⁴⁹, should have noticeably affected the climate of the high plateaus of Ethiopia.

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Archaeobacterial lipids in hot-spring microbial mats

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Modern and ancient sediments contain a multiplicity of organic compounds which record a partial history of the organisms that contributed them¹. Interpretation of this record depends on the knowledge of links between the molecular markers and their biological, notably microbial, sources. This goal is made difficult

Table 1 Isoprenyl ethers (in μg per g dry weight)* of hot-spring microbial mats in Yellowstone National Park

Spring	Temperature (°C)	pH	SO ₄ ²⁻ (mM)	Archaeobacterial content† and activity	Phytanyl	Biphytanyl					Average cyclization§
						Acyclic (0)	Monocyclic (1)	Bicyclic (2)	Tricyclic (3)	Total	
Octopus Spring (0–15 mm)	55	7.2–9.4‡	0.16	Active methanogenesis/ numerous methanogens (10 ⁷ –10 ⁸ cells cm ^{–3})	17.2	8.5	2.3	1.5	—	12.3	0.43
Octopus Spring	70	8	0.16	Less active methanogen- esis/fewer methanogens (~10 ² cells cm ^{–3})	0.4	0.3	0.3	0.2	—	0.8	0.88
Painted Pool	37	6.5	7	Methanogenesis suppressed by sulphate reduction/few methanogens (<10 ² cells cm ^{–3})	0.3	—	—	—	—	—	—
Nymph Creek	47	2.8	ND	Thermoacidophiles likely	0.2	0.2	0.2	0.6	<0.1	1.0	1.4

—, Not detected. ND, Not determined.

* Samples of 52–8,000 mg dry weight were extracted and yielded of the order of 6 mg or less of purified alkanes derived from ethers. Peaks were identified by comparison to reference mass spectra (see ref. 17) and by spectral analysis. Peak areas were determined using a V.G. Analytical Minichrom Data System, and were corrected to concentration using response factors determined by replicate injections of standards (n -C₁₈ for phytane and n -C₂₈ for biphytanes). Coefficients of variation were 0.16–0.21.

† Methane production studied by following increases in methane during anaerobic incubation of mat samples. Estimations of methanogen population density studied by most-probable-number determination. See refs 19, 20 and 22 for details.

‡ pH determined by microelectrode (see ref. 27).

§ (% monocyclic + 2 × % bicyclic + 3 × % tricyclic) × 10^{-2} after ref. 21.

|| Formulae given in Fig. 1.

by the complexity of modern sediment communities and the general lack of studies which directly tackle the relationships between the microbial inhabitants of natural systems and their associated organic compounds. We now report the first direct comparison between isoprenyl ether-linked lipids in hot-spring microbial mats and the observed populations of archaeobacteria. The particular mats examined are from Yellowstone National Park and may represent modern analogues of Precambrian communities preserved as stromatolites^{2,3}. As relatively simple microbial systems, they are suitable for validating specific molecular markers as indicators of microbial populations within a natural community. We find that the phytanyl and biphytanyl ethers reflect the measured distribution of methanogenic archaeobacteria.

An understanding of the relationships between a given biological species and its characteristic molecular markers relies on the analysis of appropriate cultures and, where possible, natural collections of that species. For many different types of land plants and algae, such procedures are relatively straightforward. For example, the link between the occurrence of 4-methylsteroids in sediments and dinoflagellates suggested by numerous sterol analyses of cultured dinoflagellates has been clearly established through direct comparison of the 4-methylsteroids of a collection taken from the water column with those of underlying sediments⁴. Similar approaches can be adopted for bacteria. For example, the occurrence of several specific acyclic isoprenoids among the free lipids of archaeobacteria⁵, a newly defined group of procaryotic microorganisms^{6–8}, grown in the laboratory was taken to suggest that they were characteristic of such organisms. Further circumstantial evidence in support of this proposition stemmed from the occurrence of these isoprenoid alkanes in sedimentary environments from which methanogenic archaeobacteria had been isolated⁹.

In addition to their free lipids, axenic cultures of representatives of the major archaeobacterial groups possess uniquely characteristic isoprenoid ethers. For example, halobacteria contain phytanyl (C₂₀)⁵ and sesterterpanyl (C₂₅)¹⁰ ethers, whereas methanogenic and other archaeobacteria (thermoacidophiles and other related organisms, such as *Thermoproteales*¹¹) produce a mixture of phytanyl and biphytanyl (C₄₀)⁵ ethers. Also, thermoacidophilic archaeobacteria can be distinguished from methanogenic archaeobacteria in that they alone are known to synthesize cyclic biphytanyl lipids⁵. All of these acyclic and cyclic isoprenoid ethers have been identified in modern and ancient sedimentary environments and fossil fuels¹² through chemical analysis. Such investigations have led to the suggestion that cyclic biphytanyl ethers are not restricted to thermoacidophiles, but may occur in methanogens¹³. An alternate interpretation is that other nonmethanogenic archaeobac-

teria are (or were) unknown inhabitants of sediments in which these cyclic biphytanyl ethers are found.

Clearly, a better understanding of the link between molecular markers and their microbial sources is desirable. For microorganisms, the traditional approach to the enrichment and isolation of axenic cultures does not ensure recovery of all the members of natural communities. Thus, the extent to which existing collections of axenic cultures (and their molecular markers) are representative of the total variety of organisms (and organic chemicals) in a given environment is not known. Lipids have been used as biochemical markers to assess directly the microbial community structure of sediments^{14,15}, but the complexity of sediment communities can seriously limit attempts to establish firm and direct relationships between organisms and their marker lipids. We have, therefore, begun investigations of such relationships in relatively simple systems, that is, hot-spring microbial mats, where high temperatures and/or acidity limit the diversity of their wholly microbial communities. However, microbial processes that control elemental cycles in these mats are similar, in principle, to those found in other aquatic systems¹⁶. These mats are also modern analogues² of the abundant Precambrian microbial communities recorded as stromatolites in the sedimentary record³. The significance of stromatolites stems from their status as a primary manifestation of life on Earth and, thus, they have a key role in the understanding of early evolution. The simplicity of the hot spring community reduces the problems of defining the relationships between organisms and molecular marker compounds. Yet, these mats are relevant analogues to the interpretation of microbial community structure in modern and ancient sediments and mats.

Samples were collected from microbial mats in three springs of Yellowstone National Park, Wyoming, USA (Table 1), using solvent-rinsed materials, immediately frozen, and lyophilized. They were treated directly with BBr₃ to release ether linked lipids as bromides, which were reduced to alkanes or D-labelled alkanes using LiAlH₄ or LiAlD₄, respectively¹⁷. Alkanes were analysed quantitatively by glass capillary gas chromatography and identified by computerized gas chromatography-mass spectrometry (C-GC-MS)¹⁸ by comparison with reference mass spectra for biphytane and its monocyclic analogue¹⁷, or by spectral interpretation. The degradative procedures released isoprenoid hydrocarbons in all samples (Table 1). The D-labelling suggested that these hydrocarbons originated from phytanyl monoethers and biphytanyl diethers.

Octopus Spring is an alkaline spring low in sulphate in which methanogenesis is active^{19,20}. Here, the highest concentrations of archaeobacterial lipids were observed in the 55 °C mat (Table 1) with phytanyl and biphytanyl ethers in approximately equal

Table 2 Depth distribution of isoprenyl ethers (in μg per g dry weight)* in Octopus Spring mat

Depth interval (mm)	Phytanyl	Biphytanyl			Total	Average Cyclization*
		Acyclic† (0)	Monocyclic (1)	Bicyclic (2)		
0-3	—	0.5	0.3	0.2	1.0	0.70
3-6	26.1	15.3	3.8	3.9	23.0	0.41
6-9	24.8	16.5	4.8	2.0	23.3	0.38
9-12	32.6	9.0	2.3	1.0	12.3	0.35
12-15	2.5	1.1	0.5	0.4	2.0	0.65

—, Not detected.

* See Table 1.

† Formulae given in Fig. 1.

abundance. This lipid distribution resembles that of *Methanobacterium thermoautotrophicum*⁵, the only methanogenic bacterium as yet isolated from this mat²⁰. The concentrations of these compounds within the mat were similar to the distribution of methanogenic bacteria and methanogenesis in the mat. They were higher at 55 °C than at 70 °C (Table 1), where methanogenesis is slight and methanogens are orders of magnitude less numerous^{19,20}.

The vertical distribution of isoprenyl ethers in the 55 °C Octopus Spring mat reveals that the highest concentrations occur in the 3-6 and 6-9 mm depth intervals (Table 2). This profile is consistent with that of methanogenesis which is most active in the 1-5 mm depth interval¹⁹. Our current method of analysis cannot, however, distinguish the complex polar lipids of living methanogenic bacteria from their degradation products retaining the isoprenyl cores. Hence, the ether cleavage products at depth in the mat may derive, in part, from the relict lipids of inactive methanogens, since methanogenesis does not occur below about 5 mm in the mat. Certainly, the isoprenoid glyceryl ethers survive in ancient sediments and even in preteroleums¹².

Cyclic biphytanyl ethers were detected in the Octopus Spring mat (Fig. 1a), although they have not been reported in *M. thermoautotrophicum* or other methanogenic bacteria. Rather, they are representative of thermoacidophilic and related archaeobacteria, but such organisms have not been isolated from mats of alkaline springs. The unexpected presence of cyclic biphytanyl ethers in this mat parallels that of the discovery of cyclic biphytanyl ethers in marine sediments and fossil fuels^{12,17}. It may be due to either lipid inputs from other archaeobacteria, or a lack of complete understanding about methanogen lipids. The simplicity of the hot-spring mat community should permit a test of the first hypothesis, and we are now developing methods to do so. The higher degree of cyclization in biphytanyl ethers observed at 70 °C than at 55 °C (Table 1) may be due either to different archaeobacterial populations, or to the influence of temperature on the lipids of a single organism. For example, *Calderiella acidophila*, a thermoacidophilic archaeobacterium, shows greater cyclization of biphytanyl ethers with increasing growth temperature²¹. Cyclization also varied with depth, but no specific trend with depth was noted.

Painted Pool is a neutral spring high in sulphate. Although methanogenic bacteria may be enriched from such high sulphate environments^{22,23}, they exist at low population density and their activity is suppressed by sulphate reduction²². These environmental features are mirrored in the low concentration of phytanyl ethers and absence of biphytanyl ethers (Table 1). Similar results have been reported for methanogen lipids in lacustrine compared to marine sediments¹⁵, where methanogenesis is limited by the level of sulphate available in the sediment²⁴.

Nymph Creek is an acid spring of 47 °C which might harbour thermoacidophilic archaeobacteria²⁵. Here, cyclic biphytanyl ethers dominated over their acyclic counterpart (Table 1; Fig. 1b), with bicyclic components the most abundant. Tricyclic biphytanyl ethers were only detected in low concentration (Table 1). Similar features have been reported for thermoacidophilic

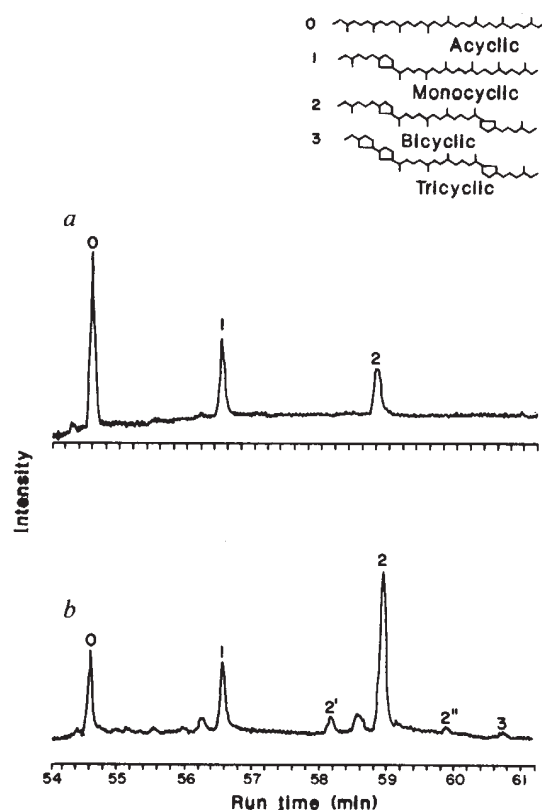


Fig. 1 Glass capillary gas chromatograms of alkanes derived from the ether cleavage procedure from: *a*, Octopus Spring 55 °C mat, 12-15 mm depth; and *b*, Nymph Creek 47 °C mat, ~0-1 cm depth. Samples were analysed on a Carlo Erba model 4160 gas chromatograph with a 50 m CPSil5CB WCOT column with H_2 carrier gas. Samples were injected on column at ~35 °C, and temperature was rapidly increased to 80 °C, then programmed to increase at 4 °C min^{-1} to 300 °C. Oven temperature was then kept isothermal at 300 °C (beginning at a run time of 55 min). Output from the flame ionization detector was processed on a V.G. Analytical Minichrom data system. Individual peaks were quantified by reference to the peak area responses of standard *n*-alkanes. *n*-alkanes were removed by urea adduction²⁶ before analysis of the sample shown in *a*. 2', 2'' = isomers of bicyclic biphytane¹².

archaeobacteria, but despite numerous attempts, such organisms, especially *Thermoplasma*, have not been isolated from this mat²⁵. The only other known thermoacidophilic archaeobacterium which might inhabit this mat is *Sulfolobus*, but apparently it cannot be enriched from acid environments at temperatures as low as that of this mat²⁵. Other related archaeobacteria (*Thermoproteales*) would not be expected¹¹ to grow at this temperature and pH. Thus, the observation of archaeobacterial lipids in this hot acid mat illustrates our incomplete understanding of the microbial inhabitants of this system and suggests the presence of archaeobacteria which have thus far eluded enrichment and isolation.

This geochemical study of a well characterized environment provides evidence for the correspondence between the distributions of specific molecular markers for methanogens and the organisms themselves. Such assessments of the organisms contributing to a given environment using molecular markers are inevitably limited by the available information on the biological occurrences of the compounds. However, where diagnostic markers are found they can provide direct evidence of the activity of specific microorganisms. In modern environments, diagnostic molecular markers may reveal the presence of otherwise unknown community members. By contrast, the absence of such molecular markers cannot always be taken as indicative of a lack of contributions from the relevant organism, since biological and chemical alterations of organic compounds may occur

after biosynthesis, but before analysis. In addition, it is inappropriate to argue that molecular markers provide a quantitative assessment of the importance of individual organisms as sources of organic matter in general, especially when the compounds are present at levels <100 p.p.m. (parts per 10⁶).

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Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor- β

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Inhibin, a specific and potent polypeptide inhibitor of the secretion of follicle-stimulating hormone (FSH)¹, of gonadal origin and thus a potential contraceptive, may constitute a missing link in the mechanism controlling the differential secretion of the pituitary gonadotropins. Inhibin-like bioactivity has been reported in various fluids and extracts of testis²⁻⁵ and in ovarian follicular fluid⁶⁻¹⁰. Although there have been several attempts to purify inhibin from

seminal plasma¹¹⁻¹³, purification from follicular fluid has been more successful (refs 14-16; for review see ref. 17). We have previously isolated two forms (A and B) of inhibin from porcine follicular fluid¹⁴. Each form comprised two dissimilar subunits of relative molecular mass (M_r) 18,000 (18K, referred to here as the α -subunit) and 14K (the β -subunit), crosslinked by one or more disulphide bridge(s). Forms A and B differ in the N-terminal sequence of their 14K subunit. Preliminary structural characterization of porcine¹⁵ and bovine¹⁶ ovarian inhibins shows that they have similar properties. Here, we have used the N-terminal amino-acid sequence data on the subunits of each inhibin to identify cloned complementary DNAs encoding the biosynthetic precursors and report that inhibins are the product of a gene family that also includes transforming growth factor- β (TGF- β) and whose structural organization is similar to that of pituitary and placental glycoprotein hormones.

The strategy for identifying clones containing coding sequences for the inhibin subunits was based on the 'long-probe' approach^{18,19}. A high-complexity cDNA library constructed in the vector λ gt10 and derived from porcine ovarian messenger RNA by oligo(dT)-primed cDNA synthesis was screened with a single 64-base synthetic oligodeoxynucleotide directed against the N-terminal amino-acid sequence of the porcine inhibin α -chain. Sequence analysis of several hybridizing cloned cDNAs confirmed correct probe identification and revealed that none of the cDNAs characterized encoded the complete structure of the α -chain precursor protein.

A second library was constructed by 3' extension on ovarian mRNA of a synthetic oligodeoxynucleotide complementary to a sequenced region encoding α precursor residues 60-64 (Fig. 1a). This library was screened with a suitable restriction fragment from a previously analysed cDNA and yielded several isolates which specified the remainder of the DNA sequences encoding the N-terminal region of the α precursor. The full sequences for the precursor protein and its cDNA are shown in Fig. 1b. The complete protein consists of 364 amino acids ($M_r \sim 39K$), of which the C-terminal 134 constitute the α -subunit. The pair of arginines preceding these 134 residues probably constitutes the site for proteolytic release of the α -subunit from its precursor. The pro-region of this precursor contains additional arginine pairs, suggesting the possible existence of other bioactive peptides within this precursor. The precursor protein sequence predicts two N-linked glycosylation sites, one within the α -chain proper. Glycosylation could account for the difference in size between that deduced from the amino-acid sequence ($M_r \sim 14.5K$) and the M_r of 18K obtained by gel electrophoresis¹⁴.

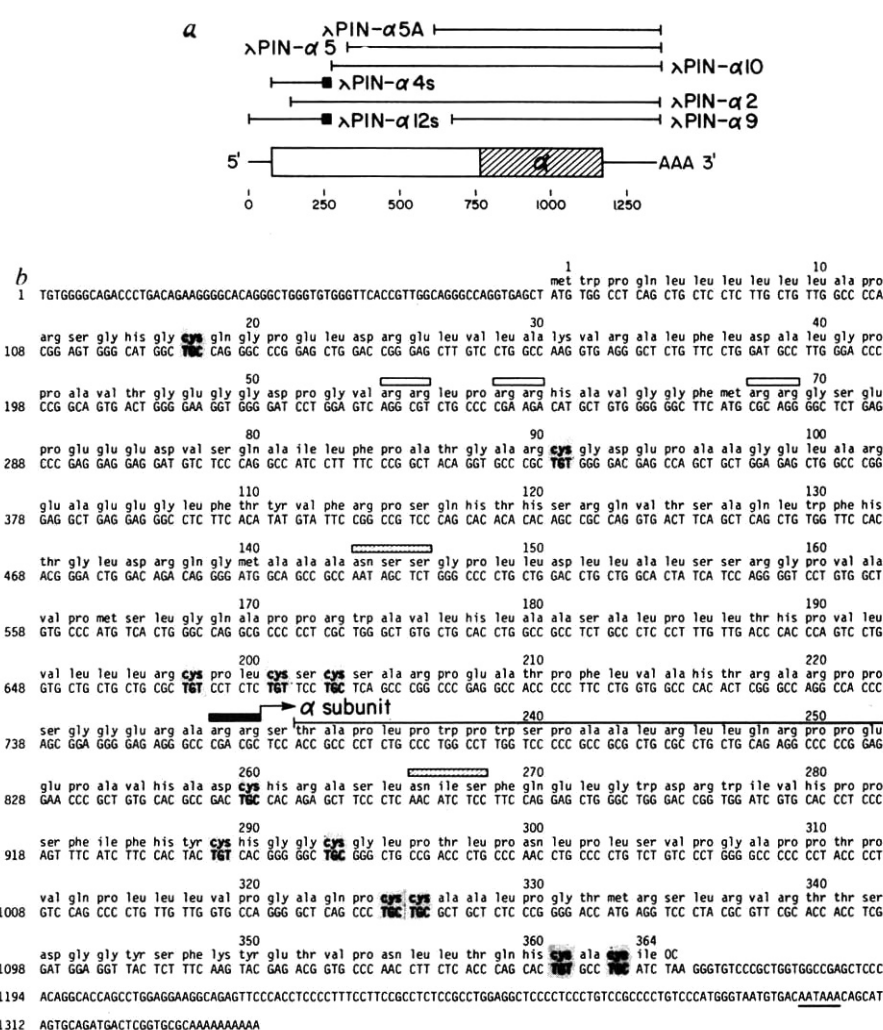
The cDNA sequences encoding the precursors of the inhibin β -subunits were obtained from the same cDNA libraries as used for the α -subunit. Overlapping cDNA clones were isolated by screening first with single long synthetic oligodeoxynucleotide probes based on the two N-terminal β -subunit sequences and subsequently with suitable restriction fragments derived from characterized cDNA clones which served as probes for 'walking' in both 5' and 3' directions (Fig. 2a).

Only four out of 2×10^5 clones from the oligo(dT)-primed library hybridized with the synthetic probe for the β -subunit of inhibin A. Three of these clones proved correct by DNA sequence analysis, but none contained a full 3'-untranslated region, as judged by the absence of a poly(A) homopolymer at their 3' ends. Unexpectedly, a higher abundance (~ 10 -fold) of inhibin β_A -subunit coding sequences was found in the cDNA library made by specific priming on α -subunit mRNA. This library was screened with the synthetic probe for the β -chain of inhibin A on our subsequently refuted working hypothesis that the α precursor mRNA might also encode the β -subunit. The high abundance of inhibin β_A cDNA in this library was later traced to a fortuitous complementarity of the specific α -chain primer to a region in the 3'-untranslated part of the β_A mRNA.

Only four cloned cDNAs encoding the β -subunit of inhibin B were found in our libraries. The sequence information

Fig. 1 Nucleotide and deduced protein sequences for porcine inhibin α -subunit precursor and its cDNA. **a**, Schematic representation of porcine α -chain mRNA. Untranslated sequences are represented by a line; coding sequences are boxed. The unfilled portion represents the coding region for the signal peptide and pro-sequences, and the cross-hatched area indicates the 134-amino-acid α -chain. The scale is in nucleotides from the 5' end of the longest cDNA clone. Overlapping cDNA clones used in sequence determination are shown above the diagram of the mRNA structure. Solid boxes at the 3' ends of λ clones indicate that these clones were obtained by specific priming. **b**, Nucleotide and predicted amino-acid sequence of the inhibin α -chain. Nucleotides are numbered at the left and amino acids are numbered throughout. The amino-acid sequence underlined was used to design a long synthetic DNA probe (see Methods). The 364-amino-acid precursor includes a hydrophobic signal sequence, a pro-region and the mature α -chain (amino acids 231–364). The proteolytic processing site Arg-Arg (black bar) immediately precedes the N-terminus of the α -chain. Several other putative dibasic processing sites present in the pro-region are indicated by open bars. The two potential N-linked glycosylation sites are shown by cross-hatched bars. The AATAAA box close to the 3' end of the mRNA is underlined.

Methods. The purification scheme used for the isolation of inhibins from porcine follicular fluid has been reported elsewhere¹⁴. Briefly, it involves successive steps of heparin-Sepharose affinity chromatography, fractionation on Sepharacyl S-200 in an acidic medium followed by four successive high-performance liquid chromatography (HPLC) steps. We identified two forms of inhibin (A and B), each of $M_r \sim 32K$, separable from one another on reverse-phase HPLC and together comprising the major proportion of inhibin-like biological activity. Another form (which may have a higher M_r , as evidenced from its earlier elution on Sepharacyl S-200 columns) was also identified but not characterized further. Isolation was monitored by *in vitro* bioassay involving a monolayer culture of dissociated rat pituitary cells²⁹. Inhibin-containing fractions were identified by their ability to inhibit the basal release of FSH but not that of LH (or other pituitary hormones), in comparison with control cultures not exposed to sample. The two subunits of inhibins A and B were separated by SDS-polyacrylamide gel electrophoresis or reverse-phase HPLC after reduction and carboxymethylation¹⁴. Microsequencing revealed that the NH₂-terminal sequences of the α -subunits of both inhibins are identical: STAPLPWPSPAA Δ RLQRPEPAV. However, the sequences of the β -subunit of inhibins A and B are clearly different, even though they are highly homologous: GLEXDGVNIXXXKKQFFVSKDIGNWDWIAP (β_A) and GLEXDGRNLXXRQFFIDFRLIGW (β_B). Both inhibins A and B specifically inhibit the basal secretion of FSH but not that of LH in the *in vitro* bioassay, with a 50% effective dose of 900 and 450 pg ml⁻¹, respectively. Polyadenylated mRNA was prepared from freshly frozen porcine ovaries³⁰. An oligo(dT)-primed cDNA library of $\sim 6 \times 10^6$ clones in λ gt10 (ref. 31) was prepared from 5 μ g poly(A)⁺ mRNA as described elsewhere³². Approximately 1×10^6 unamplified cDNA clones were screened with α -subunit oligonucleotide. 5'-ACCGCCCTTTGGCTTGGCTCCCTGCTGCTCTGAGACTGCTGCAGAGACCTC-CTGAGG-3', based on the amino-acid sequence overlined in **b**. Hybridization was carried out with the phosphorylated ³²P-labelled probe in 5 $\times$ SSC, 40% formamide at 37°C. Filters were washed at 50°C with 1 $\times$ SSC, 0.1% SDS. Approximately 500 hybridization-positive clones were obtained, 12 of which were purified and examined for insert size. The EcoRI inserts of five of these (λ PIN- α 2, α 5A, α 5, α 9, α 10) were subcloned into M13 derivatives³³ and sequenced by the dideoxy chain-termination method³⁴. A specifically primed library was prepared by priming 5 μ g of poly(A)⁺ mRNA with the oligonucleotide 5'-CCCCACAGCATGCTT-3' (complementary to nucleotides 248–263) and subsequent cloning into λ gt10. Approximately 2×10^5 clones of the 1×10^6 clones obtained were screened with the 5' 104 base pair (bp) EcoRI/BamHI fragment prepared from λ PIN- α 2. Of the 170 hybridization-positive clones obtained, 12 were purified and 2 (λ PIN- α 12s, α 4s) were sequenced by the dideoxy method. The complete nucleotide sequences of the α -subunit cDNAs were obtained by subcloning various restriction fragments from the different λ isolates into the M13 phage derivatives^{33,34}. Compressed sequence ladders were made unambiguous by the use of deoxyinosine mixes, in severe cases in the presence of *Escherichia coli* single-stranded binding protein (Pharmacia).



obtained from these clones failed to reveal the complete structure of the corresponding precursor protein and its cDNA. Figure 2b compares our sequences of cDNAs and deduced protein structures for the precursors of the β -subunits. The nucleotide sequence of inhibin β_A -subunit cDNA is ~ 3.6 kilobases (kb) long. A methionine codon at nucleotides 34–36 initiates a long open reading frame specifying a protein of 424 amino acids ($M_r \sim 47.5K$), of which the C-terminal 116 residues represent the β -subunit proper. The β_A -subunit is preceded by five consecutive arginines, at which it is presumably cleaved proteolytically from the precursor. Similar to the α -subunit precursor, this β precursor contains additional pairs of basic residues which might constitute other precursor cleavage sites resulting in the possible release of hitherto unknown biologically active peptides. It also contains one site for N-linked glycosylation in the pro-region (Asn, residue 165).

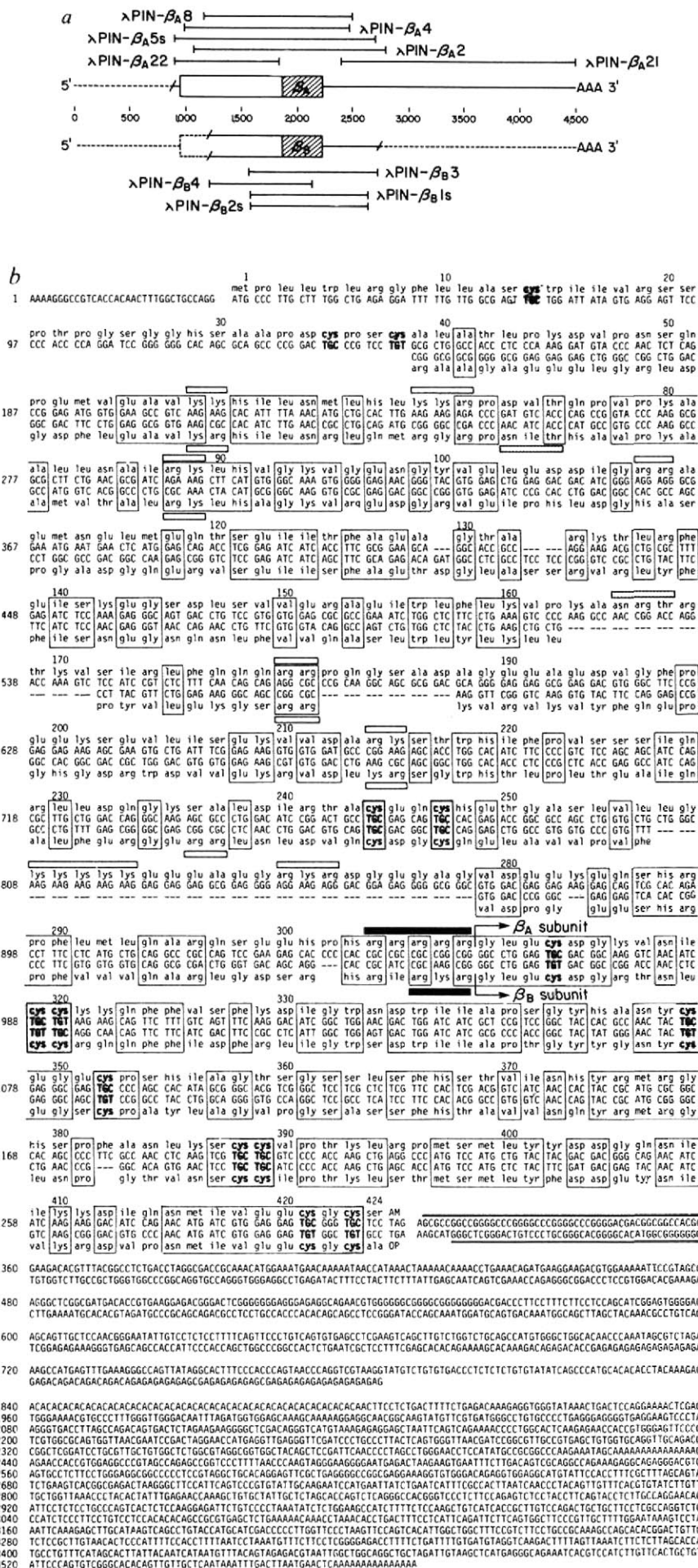
The deduced protein sequence for the β -subunit of inhibin B is one amino acid shorter than the β_A -subunit and shows 70%

homology with the β_A -subunit sequence. Sequence homology of a lesser degree is also found in the pro-region of both precursors. Interestingly, an extremely purine-rich sequence rarely seen in coding regions but present in the cDNA encoding the inhibin β_A precursor and resulting in a curious amino-acid sequence, is not found in the cDNA coding for the homologous β_B precursor. The absence of this sequence results in a deletion of 22 amino-acid residues from the β precursor of inhibin B when protein sequences are aligned for maximum homology. Such alignment brings about a perfect match in the cysteine positions of both precursors.

Ovarian total and polyadenylated RNAs were analysed by the Northern procedure to assess size and relative abundance of the mRNAs encoding the peptide subunits of both forms of inhibin. DNA sequences encoding the divergent pro-regions of β_A and β_B were used as hybridization probes in stringent conditions. Analysis showed (Fig. 3) that α and β mRNAs are of different size and abundance, confirming results obtained from

Fig. 2 Inhibin β -subunits; nucleotide sequence of cDNAs and predicted primary structure of precursors. **a**, Schematic representation of the porcine β_A and β_B -subunit mRNAs with coding sequences boxed. The β_A and β_B -subunits (cross-hatched boxes) are encoded towards the 3' end of the coding sequences. The 3'- and 5'-untranslated regions are shown as a line. The length of the 5' end of β_A mRNA and the 5'- and 3'-untranslated regions of the β_B -subunit mRNA are inferred from the size of the mRNAs (Fig. 3) and the obvious similarity of β_B to β_A mRNA. Regions of the cDNAs not obtained by cloning are shown as dashes in the diagram. The relative positions of the overlapping oligo(dT)-primed cDNA clones and the randomly primed clones (λ PIN- β_A 5s, λ PIN- β_A 1s and λ PIN- β_B 2s) are indicated. The scale is in nucleotides from the 5' end of the 4.5-kb mRNA. **b**, Sequence comparisons. Nucleotide numbers on the left refer to those of cloned β_A cDNA sequences. Amino-acid numbers are for β_A precursor residues. The N-termini of the β -subunits, which are preceded by basic processing sites (black bars), are indicated by arrows. The β_B sequence is shown underneath the β_A sequence and aligned with it for maximum homology. Regions containing identical amino-acid sequences are boxed. Cysteine residues are shaded, possible processing sites are indicated by open bars, and potential glycosylation sites are shown by cross-hatched bars. A highly G+C-rich region 3' to the termination codon is underlined in one and overlined in the other sequence, and the AATAAA polyadenylation signal is underlined in the β_A -subunit sequence. There was one nucleotide difference between λ PIN- β_B 8 and the other clones covering this area: a G $\rightarrow$ A change at nucleotide 868 results in a change of amino-acid 278 from a glycine to a serine. Another polymorphism was observed at amino-acid 206 in the β_B -subunit sequence shown, where the T $\rightarrow$ C change found only in λ PIN- β_B 1 would result in a Phe $\rightarrow$ Leu change.

Methods. Approximately 2×10^5 oligo(dT)-primed ovarian cDNA clones were screened with the 5' 32 P-labelled β_A oligonucleotide, 5'-AAGAAGCAGTTC-TTTGTGTCCTTCAAGGACATTTGGCTGGG-ATGACTGATCAATTC-3', based on the amino-acid sequence (residues 321-339). Four hybridization positives were obtained, of which three proved to contain β_A coding sequences (λ PIN- β_A 2, β_A 4, β_A 8). A 5'-end 157-bp *EcoRI*/*HindIII* (nucleotides 158-298) fragment and a 3'-end 221-bp *EcoRI*/*Pst* fragment (nucleotides 1,681-1,885) derived from λ PIN β_A 2 were used to screen 2×10^6 oligo(dT)-primed cDNA clones and 2×10^5 clones from the specifically α -chain primed library. Of the 16 clones analysed in detail, 2 were found to have longer 5' ends (λ PIN- β_A 5s, β_A 22) and 1 (λ PIN- β_A 21) contained the entire 3'-untranslated region. The *EcoRI* restriction sites do not refer to sequences present in the cDNA but are all contained within the cDNA adaptor fragment and thus add an extra 16 nucleotides to the above fragment sizes. Porcine inhibin β_B -subunit cDNA clones were isolated by screening 2×10^5 clones from the specifically primed library with the β_B oligonucleotide, 5'-GGCCTGGAGTGTGATGGGAGACCAACCTGTCTGCGCCAGCAGTTCATCATCGATTTCAGGCT-3', which was based on the NH₂-terminal sequence described in Fig. 1a legend. Positive clones were further screened with the oligonucleotide inosine probe 5'-AATCTATATAA-ACTGCTG-3' containing deoxyinosine³⁵, which covers all the possibilities in the noncoding strand for the amino-acid sequence QFFIDF. Two clones (λ PIN- β_B 1s, β_B 2s) were sequenced and found to code for the β_B -subunit. A 231-bp *EcoRI*/*Sma* (nucleotides 406-630) fragment was isolated from λ PIN- β_B -1 and used as a hybridization probe to screen 2×10^6 oligo(dT)-primed cDNA clones. Two positives were obtained (λ PIN- β_B 3, β_B 4). The nucleotide sequence of these overlapping clones was used to construct the sequence shown. All sequences were obtained by subcloning specific fragments into M13 phage vectors^{33,34}.



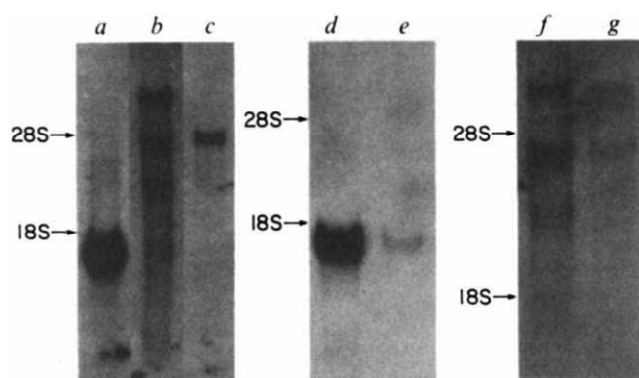


Fig. 3 Northern analysis of mRNA for inhibin subunits α , β_A and β_B . Lanes *a*, *b*, *c*, *d* and *f* contain poly(A)⁺ RNA and lanes *e* and *g* contain total RNA. The positions of the 28S and 18S ribosomal RNAs are indicated by arrows. Lanes *a*, *d* and *e* were hybridized with an α -subunit cDNA probe, lanes *b*, *f* and *g* with a β_A -subunit-specific probe, lane *c* with a β_B -subunit-specific probe. The α -subunit mRNA is ~1.5 kb long, the β_A -subunit mRNAs are ~4.5 and 7.2 kb, and the β_B -subunit mRNA is ~4.5 kb long. The hybridizations shown in lanes *a*, *b* and *c* were performed with probes of approximately equal length and specific activity in order to judge relative mRNA levels.

Methods. Polyadenylated RNA (2 μ g, lanes *a*, *b*, *c*, *f*; 8 μ g, lane *d*) and total RNA (10 μ g, lanes *e*, *g*) were electrophoresed into a formaldehyde-1.2% agarose gel containing formaldehyde as a denaturant³⁶ and blotted onto a nitrocellulose filter³⁷. The following ³²P-labelled cDNA fragments were used as hybridization probes. Lane *a*, 258-bp *Eco*RI/*Sma*I (nucleotides 131-371) from α -subunit cDNA λ PIN- α 2; *b*, 157-bp *Eco*RI/*Hind*III (nucleotides 158-298) from β_A -subunit cDNA λ PIN- β A2; *c*, 231-bp *Eco*RI/*Sma*I (nucleotides 406-630) from β_B -subunit cDNA λ PIN- β B1; *d*, *e*, *Eco*RI insert of λ PIN- α 2; *f*, *g*, *Eco*RI insert of λ PIN- β A4. Filters were washed for 2 h with three changes of 0.1 $\times$ SSC, 0.1% SDS at 60 °C.

cDNA cloning. From their respective band intensities, the α precursor mRNA is estimated to be in at least 10-fold higher abundance than the mRNA for the β_A precursor, and ~20-fold higher than the β_B precursor mRNA.

Using ribosomal RNAs as size standards, the α precursor mRNA, a single species, is found to be ~1,500 nucleotides long, in good agreement with the cloned cDNA sequence (Fig. 1*b*). β_A precursor mRNA sequences are represented by two main species of ~4.5 and ~7.2 kb. The uniformly higher intensities of α and β_A species in polyadenylated than in total RNA (Fig. 3, lanes *d*–*g*) suggest that the β_A 4.5-kb species does not represent an artefact created by 28S RNA hybridizing to the cDNA probe. The β_A precursor cDNA sequences shown in Fig. 2*b* are therefore thought to be complementary to this 4.5-kb mRNA. The difference in size from the 3.6-kb-long cloned cDNA is attributed to missing 5' sequences. Unusually long 5'-untranslated regions are not without precedent^{20–22}. A differential splicing event or choice of a different polyadenylation signal might explain the existence of the 7.2-kb mRNA^{23,24}. The β_B precursor is encoded by a single mRNA of ~4.5 kb and is present at approximately half the level of the two β_A mRNAs.

The mature inhibin α - and β -subunits contain seven and nine cysteine residues respectively. Alignment of these residues shows that both types of subunits share a similar cysteine distribution and that some sequence homology exists around these residues (Fig. 4*a*), suggesting that α - and β -subunits are derived from one ancestral gene. Surprisingly, significant homology was found between the β -type sequence and the recently determined primary structure of human TGF- β ²⁰. As outlined in Fig. 4*b*, these peptides are of nearly equal length (inhibin β_A subunit, 116 residues; β_B subunit, 115; TGF- β , 112) and show a strikingly similar distribution of their nine cysteine residues. Using this cysteine 'pattern' for alignment, the β_A and TGF- β sequences

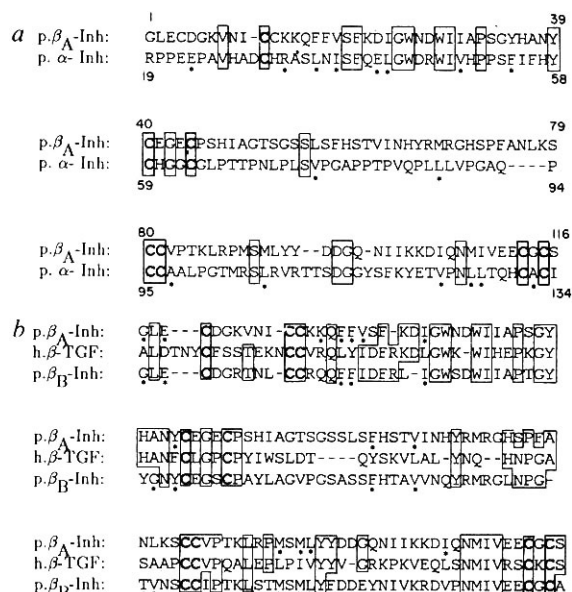


Fig. 4 Sequence homologies of inhibin subunits and TGF- β . *a*, Comparison of porcine inhibin β_A and α -subunit sequences. Numbers refer to the mature subunit sequences. *b*, Comparison of human TGF- β ²⁰ and porcine inhibin β -subunit sequences. Amino acids are shown in the one-letter code. Identical residues are boxed with cysteine residues shaded. Asterisks denote conservative changes.

have an additional 33 residues in identical positions and show conservative changes in more than 10 places. Similar homologies are seen on comparison of the β_B and TGF- β sequences. The β -subunit of inhibin is unlikely to be the porcine equivalent of human TGF- β , as there is almost absolute homology between human and murine TGF- β (R. Derynck, personal communication). Sequence homology extends into the pro-region where the inhibin β and TGF- β precursors²⁰ show similar hydrophobicity profiles (data not shown). Certain features of their cDNAs, in particular the highly G + C-rich sequence following the termination codons, may further attest to a common origin. Our findings strongly indicate that both inhibin subunits and TGF- β belong to one gene family and are biologically active as dimeric structures in which the subunits are linked by disulphide bridges^{14,25}.

The structural organization of porcine ovarian follicular fluid shares some intriguing features with the pituitary and placental glycoprotein hormones, FSH, luteinizing hormone (LH), thyroid stimulating hormone and chorionic gonadotropin²⁶. Like these molecules, inhibins A and B are high-*M_r* glycoproteins and have two subunits, one of which is common to both forms, the other variable, all encoded by different mRNA species. The mRNA encoding the common (α) subunit in both structures is in substantially higher abundance than the corresponding β -subunit mRNA, suggesting a similarity in the regulation of biosynthesis of each group of hormones²⁷. In the abovementioned hormones, the 'variable' (β) subunit confers the different biological specificities of each molecule, while the 'constant' (α) subunit presumably has a crucial role in folding and overall conformation of the complex²⁶. We have no evidence for differences in the biological properties of inhibins A and B. Unlike the known glycoprotein hormones, the two chains of both inhibins A and B are linked by disulphide bridges, a situation more closely resembling the immunoglobulins²⁸.

Regarding biosynthesis, each inhibin chain is released through proteolytic processing of a larger precursor, the α - and β -subunits representing the C-terminal region of each polypeptide. The discrepancy in size between the larger subunits of bovine¹⁶ and porcine inhibins can be readily explained by a different processing of their precursors.

The precursors for the porcine inhibin subunits have several common structural features: they carry the subunits at their C-termini, are similar in total size, have typical signal sequences at their N-termini and show a remarkably similar distribution of cysteine residues. In fact, closer analysis reveals significant sequence homology between the α - and β -subunits suggestive of an origin from distantly related genes. This, too, is a feature of the subunits constituting the pituitary and placental glycoprotein hormones²⁶.

Of considerable interest is the high homology of inhibin β -subunits with TGF- β , which points to an evolutionary link between the corresponding genes. It is intriguing that members of this distinctive gene family should be involved in such seemingly unrelated activities as the control of reproduction and cellular growth. What determines the activities of their respective protein products?

A clue may be found in the fact that TGF- β is active in a homodimeric form more homologous in amino-acid sequence to the β -subunits than to the α -subunit of inhibin. This fact and the analogy with the glycoprotein hormones noted above, direct attention to the β -subunits as the possible source of biological specificity of inhibin. Chain recombination experiments to generate novel heterodimers between inhibin and TGF- β subunits could test this hypothesis. Moreover, it is tempting to speculate that inhibin could function as a paracrine or autocrine growth regulator within gonadal tissue.

The prediction of the full inhibin structures from cloned cDNA sequences has allowed us to obtain specific antibodies against synthetic peptides based on these sequences, which are currently used to study inhibin regulation. The availability of cloned DNA sequences for inhibins will help to characterize the expression of these genes in gonads and possibly other tissues. More importantly, the recombinant expression of these sequences will allow the elucidation of their physiological role at the gonadal, pituitary and hypothalamic level.

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Epidermal growth factor receptor occupancy inhibits vaccinia virus infection

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Vaccinia virus encodes VGF, an early protein of relative molecular mass 19,000 (19K) which, from amino-acid residues 45 to 85, is homologous in 19 residues to epidermal growth factor (EGF), and transforming growth factor- α (TGF- α)^{1–3}. The conserved sequence includes a region of high homology (6 out of 10 amino acids) from residues 71 to 80, corresponding to the third disulphide loop of both EGF and TGF- α . This region has recently been shown to contain a binding region of TGF- α for the EGF receptor⁴, and this raises the question of whether vaccinia virus utilizes the EGF receptor in order to bind to and infect cells. We now show that occupancy of the EGF receptor inhibits vaccinia virus infection. Inhibition is observed in a dose-dependent fashion by pre-treatment with either EGF or synthetic decapeptide antagonists of EGF's mitogenic activity which correspond to the sequence of the third disulphide loop of VGF or TGF- α . The relative ability of the peptides to inhibit vaccinia virus infection parallels their binding affinity to the EGF receptor.

In order to test the concept that vaccinia virus makes use of the EGF receptor to infect cells, the effects of EGF and peptide antagonists of EGF were studied in a viral replication assay. Treatment of murine L-cells with EGF prior to the addition of vaccinia virus (strain WR; American Type Culture Collection) for a 1-h adsorption period inhibited viral infection in a dose-dependent manner, as monitored by a plaque reduction assay (Fig. 1). Further addition of EGF after the initial 1-h adsorption period and for the 4-day duration of the plaque assay resulted in a slight additional decrease in viral plaque number. Similarly, addition of synthetic decapeptide antagonists^{4,5} corresponding to the third disulphide loop of VGF or TGF- α (Fig. 2) resulted in a dose-related reduction in the number of viral plaques (Fig. 1). Further analysis of inhibition of single-cycle vaccinia virus replication by EGF of the synthetic decapeptides showed that whereas inhibition was achieved by EGF or decapeptides when the peptides were present before and during viral adsorption, no inhibition was observed when the peptides were added 2 h after viral adsorption. The additional decrease in viral plaque formation in the multi-cycle plaque assay when the peptides were also added 1 h after initial viral adsorption (Fig. 1) can be attributed both to inhibition of adsorption which was not complete in 1 h (which was confirmed in single-cycle virus yield experiments) and inhibition of viral adsorption in subsequent replication cycles.

The ability of EGF and the synthetic peptides to inhibit vaccinia virus replication correlated with their affinity for the EGF receptor on the L-cells (Fig. 3). EGF inhibited vaccinia virus plaque formation by approximately 50% at a concentration of 10^{-8} M (compared with 10^{-9} M for 50% maximal binding). The synthetic decapeptide antagonists (N and C terminal capped) which were approximately 10^3 times lower in EGF receptor

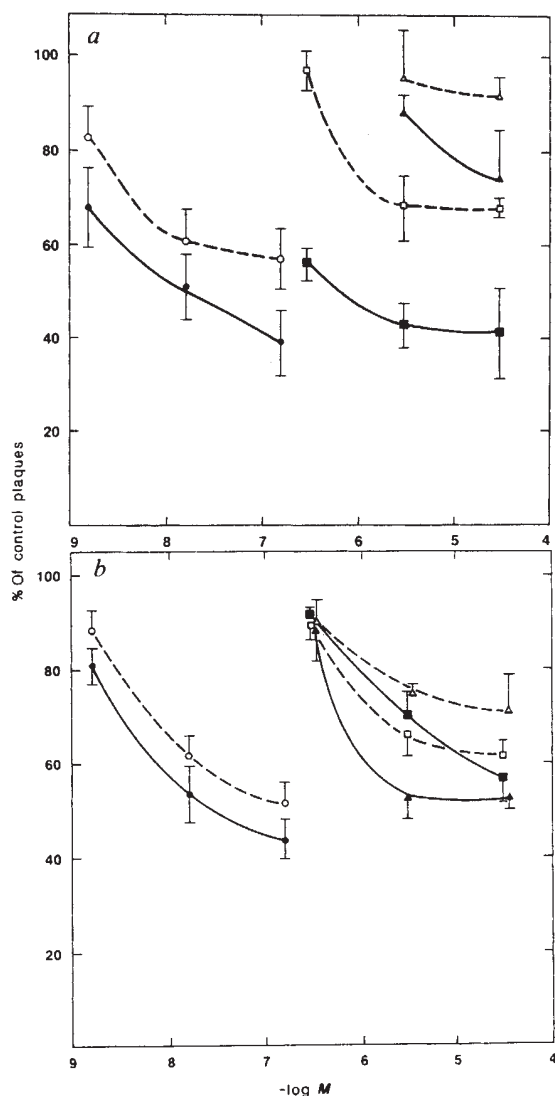


Fig. 1 Inhibition of vaccinia virus plaque formation by EGF and related synthetic decapeptides. Plaque reduction of vaccinia virus in L fibroblasts was performed as described elsewhere¹². EGF was purified from mouse submaxillary glands⁴. Peptide compounds were added to cells 3 h before viral adsorption as well as being left in the presence of cells during a 1-h viral adsorption period (open symbols); or were added both before and during viral adsorption as well as in the agar overlay after aspiration of the inoculum virus (solid symbols). Values plotted are the mean of three replicates $\pm$ s.d. *a* = $\circ$, $\bullet$, EGF; $\square$, $\blacksquare$, acetyl TGF- α (34-43)-NH₂; $\triangle$, $\blacktriangle$, H-TGF- α (34-43)-OH (virus control was 38.8 ± 4.1 plaques, average of 6 replicates). *b* = $\circ$, $\bullet$, EGF; $\square$, $\blacksquare$, acetyl VGF(71-80)-OMe; $\triangle$, $\blacktriangle$, acetyl VGF(71-80)-NH₂ (virus control was 33.7 ± 1.0 plaques, average of 6 replicates).

affinity, showed a similar decreased potency relative to EGF for the inhibition of vaccinia virus replication. The corresponding 'free acid' decapeptide, which was less effective in binding to the EGF receptor, was similarly less effective in preventing vaccinia virus plaque formation. The fact that complete inhibition of vaccinia virus infections was not obtained at concentrations of EGF or peptides that were saturating in terms of binding to the EGF receptor suggests that, although initial interaction of VV with the EGF receptor may lead to productive infection, it is not the only pathway by which VV can infect L cells. Recently Stroobant *et al.*¹³ reported that VV was able to infect NR-G cells which lack detectable EGF receptors.

Specificity of the inhibition of vaccinia virus replication by EGF and the homologous synthetic peptides was confirmed by (1) lack of inhibition of vaccinia viral plaque formation or

Amino-acid no.													
VGF	---	71	C	S	H	G	Y	T	G	I	R	C	---
HuEGF	---	33	C	V	V	G	Y	I	G	E	R	C	---
MuEGF	---	34	C	V	I	G	Y	S	G	D	R	C	---
HuTGF- α	---	34	C	H	S	G	Y	V	G	A	R	C	---
TGF- α (34-43) free acid			C	H	S	G	Y	V	G	A	R	C	OH
Acetyl TGF- α (34-43) ethylamide	Ac		C	H	S	G	Y	V	G	A	R	C	NHEt
VGF(71-80) free acid			C	S	H	G	Y	T	G	I	R	C	OH
Acetyl VGF(71-80) amide	Ac		C	S	H	G	Y	T	G	I	R	C	NH ₂
Acetyl VGF(71-80) methyl ester	Ac		C	S	H	G	Y	T	G	I	R	C	OMe

Fig. 2 Peptide sequence of EGF, VGF and the TGF- α third disulphide loop, and sequences of the synthetic decapeptides. Sequence homologies between all peptides are boxed. TGF- α peptides and VGF peptides were synthesized using solid-phase techniques as described for the TGF- α peptides^{4,5}.

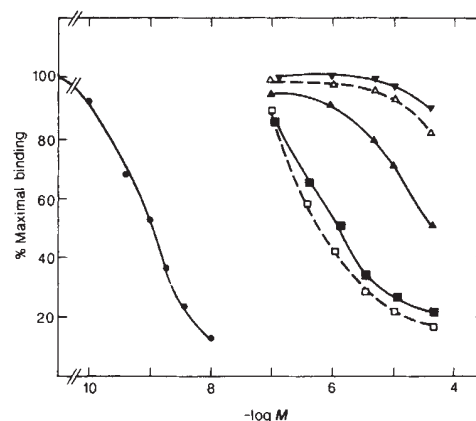


Fig. 3 Inhibition of binding of ¹²⁵I-EGF to L-cells. EGF was radioiodinated with Enzymobeads (Bio-Rad) to 120,000 c.p.m. per ng protein⁴. Receptor binding on L-cells was performed as previously described for A431 cells⁴. Nonspecific binding (determined in the presence of 100 times excess cold EGF) was $\leq 15\%$ of the specific binding; approximately 40,000–50,000 specific high-affinity binding sites were found per cell. L-cells were incubated at 4 °C with 1 nM ¹²⁵I-EGF in the presence of EGF($\bullet$), acetyl TGF- α (34-43)-NH₂($\square$), H-TGF- α (34-43)-OH($\triangle$), acetyl VGF(71-80)-OMe($\blacksquare$), acetyl VGF(71-80)-NH₂($\blacktriangle$) or H-VGF(71-80)-OH($\blacktriangledown$). Average of 3 replicates plotted; s.d. $\leq 5\%$.

single-cycle virus yield by two unrelated polypeptides which bind to their respective receptors on L-cells, that is, insulin ($1 \mu\text{g ml}^{-1}$) or bovine growth hormone ($1 \mu\text{g ml}^{-1}$); and (2) no inhibition of two unrelated viruses by EGF or synthetic peptide (an RNA virus, vesicular stomatitis virus, Indiana strain, on L-cells; or a DNA virus, herpes simplex virus type II, strain G, on Vero cells).

It is noteworthy that EGF, which is an agonist in stimulating cellular DNA synthesis, and the synthetic decapeptides, which are antagonists of the mitogenic effect of EGF^{4,5}, both appear to inhibit vaccinia virus receptor interaction, with subsequent inhibition of viral replication. These results suggest that (1) it is likely that the inhibition occurs through blocking of the receptor site utilized by the virus and not by an indirect phenomenon after activation of the EGF-receptor system, and (2) a mitogenic effect of the native vaccinia virus 19K protein is not an obligatory requirement for viral replication. Recently, a vaccinia virus 25K protein (isolated from supernatants of

vaccinia virus-infected cells) was shown to bind to the EGF receptor and to have cellular mitogenic activity⁶. It remains to be demonstrated whether this protein corresponds to the 19K protein¹⁻³ bearing homology to TGF- α and EGF. It is possible that the growth factor secreted by vaccinia virus-infected cells may account for cellular hyperproliferation as previously suggested^{1,6}, as well as contribute to the self-limiting nature of vaccinia virus lesions.

The cellular receptors for certain viruses have been identified as being receptors for various other specific ligands. For example, Epstein-Barr virus infects B lymphocytes via the complement receptor CR2 (ref. 7); rabies virus may utilize the acetylcholine receptor⁸; the human T-cell lymphotropic virus type III (HTLV-III) receptor on T lymphocytes contains the CD4 (or T4) antigen^{9,10}; and the reovirus receptor has recently been identified as the cellular β -adrenergic receptor¹¹ (G. Gaulton, personal communication). Our results now provide another example of a virus that appears to utilize a cellular receptor specific for a physiological ligand.

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Characterization of receptors for human tumour necrosis factor and their regulation by γ -interferon

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Tumour necrosis factors, TNF- α and TNF- β (previously called lymphotoxin), are the products of activated monocytes and lymphocytes, respectively, and both have recently been purified, sequenced and cloned by recombinant DNA methods¹⁻⁵, revealing 35% identity and 50% homology in the amino-acid sequence. Both proteins have been found to be specifically toxic to many tumour cells. Furthermore, it has been reported that various interferons are synergistic with TNF for anti-tumour effects *in vitro*⁶⁻⁸, while activities attributed to the two proteins have also been shown to necrotize various tumours *in vivo*^{2,3,9}. We have now prepared ¹²⁵I-labelled highly purified recombinant human TNF- α to study in detail its binding to the human cervical carcinoma cell line ME-180. Our results indicate that there is a single class of specific high-affinity receptors for TNF on this cell line which has a K_d of about 0.2 nM and an average of 2,000 receptor sites per cell. The binding of labelled TNF- α to these cells can be inhibited by both TNF- α and TNF- β but not by γ -interferon (IFN- γ). However, preincubation of cells with IFN- γ increases the total number of TNF receptors two to threefold without any significant change in the affinity constant. This is the first report that TNF- α

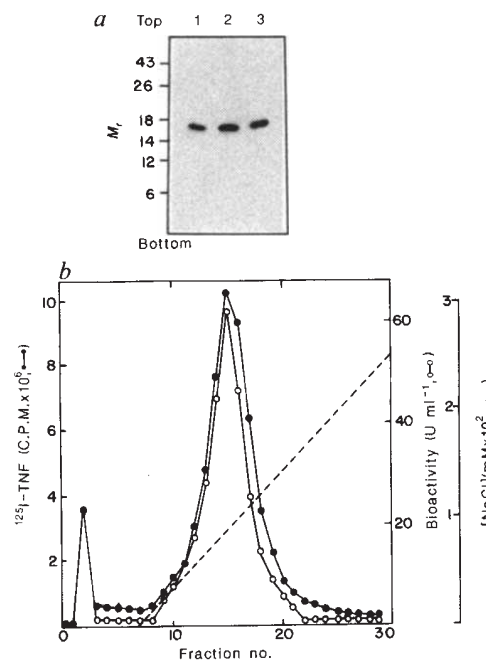


Fig. 1 SDS-polyacrylamide gel electrophoresis (a) and Mono Q-fast protein liquid chromatography of iodinated TNF- α (b). The autoradiographs shown in a represent samples from three different iodinations (lanes 1-3). About 10,000 c.p.m. of ¹²⁵I-TNF were analysed. The M_r s ($\times 10^3$) of protein standards run in parallel are indicated.

Methods. Purified TNF (10 μ g) was radioiodinated by the iodogen method¹⁰. 0.1 M phosphate buffer (pH 7.4) was added to a glass tube coated with 5 μ g of iodogen (Pierce) and incubated with 1 mCi of Na¹²⁵I for 10 min at 4 °C. Then the mixture was transferred to a tube containing TNF- α and allowed to react for 5 min at 4 °C. The reaction was stopped by removal of the soluble material which was separated from free iodide by filtration with Sephadex G-25 that had been equilibrated with 0.1 M phosphate buffer containing 0.1% gelatin. Sephadex G-25-purified material was characterized further on a Mono Q-column equilibrated in 25 mM Tris pH 7.4 containing 0.05% Tween 20 (b). Linear salt gradients of 0-0.25 M NaCl in equilibration buffer were used for elution of ¹²⁵I-TNF. The column was eluted at a flow rate of 1 ml min⁻¹ and 2 ml per fraction was collected. All these operations were carried out at room temperature. The column fractions were stored at 4 °C and analysed for radioactivity and cytolytic activity. The latter was measured by the lysis of actinomycin D-treated mouse fibroblast L929 cells (30,000 cells per microtitre well) plated in monolayer and quantitated by dye uptake⁷. The protein concentration of iodinated TNF was determined by immunoassay for TNF- α . Column fractions were also analysed by electrophoresis on 15% polyacrylamide slab gels¹⁷.

and - β share a common receptor and that the receptors can be up-regulated by interferon. Our results may explain previous observations regarding similar biological activities observed for these two cytotoxic proteins and also their synergistic action with interferons.

TNF- α and - β have been cloned and expressed by recombinant DNA methods in *Escherichia coli*. The proteins were purified to homogeneity from bacteria either by immunoaffinity purification using monoclonal antibodies or by ion-exchange chromatography as described previously¹⁻⁵. Both proteins were homogeneous by the criteria of amino-terminal sequence analysis and SDS-polyacrylamide gel electrophoresis. TNF- α and - β have relative molecular masses (M_r s) of 17,000 and 15,500, respectively, and specific activities of $\sim 100 \times 10^6$ units per mg as determined by lysis of actinomycin D-treated mouse L929 cells⁷.

The labelling of TNF- α and - β by chloramine-T, lactoperoxidase and Bolton-Hunter reagent methods provided ¹²⁵I-TNF with low specific activity and resulted in some cases in a >90% decline in biological activity. However, when TNF- α

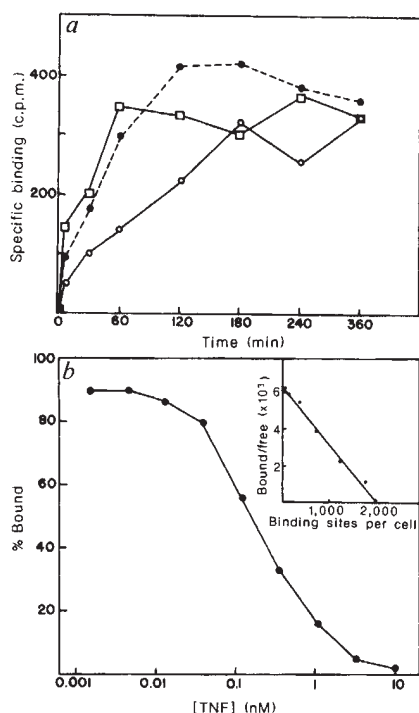


Fig. 2 *a*, Time course of ^{125}I -TNF binding at 4 °C (○), 23 °C (●) and 37 °C (□) and *b*, competition curve of labelled bound TNF- α with unlabelled TNF- α . For each time point in *a*, 0.2×10^6 ME-180 cell monolayers were incubated in a final volume of 0.5 ml of McCoy's 5A medium containing 10% fetal bovine serum at the indicated temperature with 0.2×10^6 c.p.m. of ^{125}I -TNF- α in the presence and absence of 100 nM unlabelled TNF- α . Each data point represents the mean of triplicate determinations. After incubation, cell monolayers were washed three times with the incubation buffer, solubilized with 2% SDS and cell-bound radioactivity was determined in a γ -counter. In *b*, cells were incubated at 37 °C for 2 h with 0.2×10^6 c.p.m. of ^{125}I -TNF- α and indicated amounts of unlabelled TNF- α . Nonspecific binding was $\leq 10\%$ of total binding. The data are plotted as specific binding against concentration of unlabelled TNF- α . Inset, a Scatchard plot of the data.

was labelled by the iodogen method¹⁰, a fully biologically active ^{125}I -TNF- α was obtained which was stable for more than 4 weeks. ^{125}I -TNF- α had a specific activity of ~ 804 Ci per mmol protein, and $\sim 50\%$ incorporation of the label into the protein was observed. Approximately 50% of the TNF- α molecules were labelled, assuming monoiodination by this method. TNF- α contains seven tyrosine residues. Digestion of ^{125}I -TNF- α by trypsin (5% w/w) provided a single radioactive peptide. The amino-acid sequence of this peptide indicated that the tyrosine residue at position 87 was radiolabelled.

The product of this iodination reaction showed a single- M_r component of $\sim 17,000$ (TNF- α has a M_r of 17,100 as calculated from the protein sequence) when analysed by gel electrophoresis (Fig. 1*a*). Fractionation of ^{125}I -TNF- α by gel filtration chromatography on a TSK column also provided a single peak of radioactive protein which co-eluted with the biological activity (data not shown). The apparent M_r of labelled TNF- α by gel filtration was found to be $\sim 20,000$, similar to that determined for unlabelled recombinant TNF. A single peak of radioactive protein also co-eluted with biological activity when chromatographed by Mono Q-fast protein liquid chromatography (Fig. 1*b*), showing a retention time similar to that of unlabelled TNF.

We investigated the interaction of ^{125}I -TNF- α with cellular binding sites by incubating it with monolayers of human tumour cells in tissue culture. After incubation, the cells were washed three times with incubation medium, solubilized with 2% SDS and the radioactivity bound to the cell monolayer was measured. To avoid a species-specific barrier, human tumour cells were used to determine the binding of human TNF- α . It has been

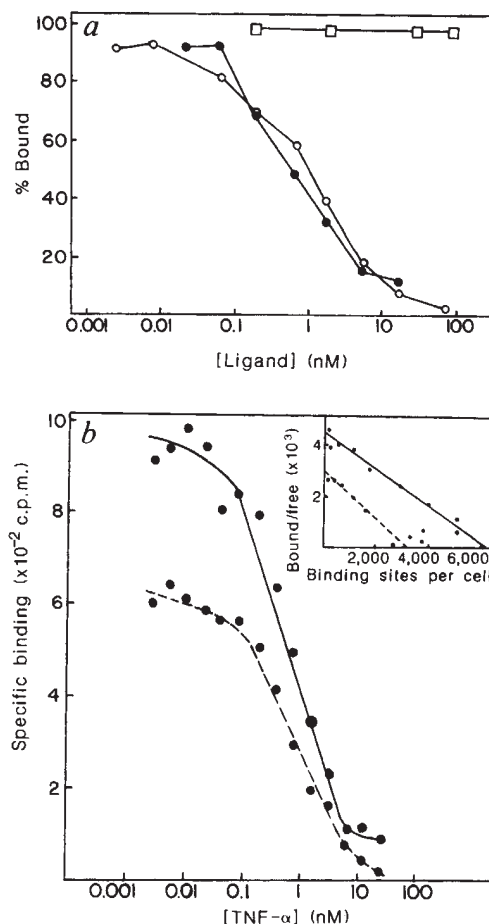


Fig. 3 *a*, Competition of TNF- α , TNF- β and IFN- γ with ^{125}I -TNF- α for binding to ME-180 cells; *b*, competition of TNF- α with ^{125}I -TNF- α for binding to ME-180 cells before and after exposure to IFN- γ . Incubation conditions for *a* were as described for Fig. 2*a*, with the addition of the indicated concentrations of the following: TNF- α (●), TNF- β (○) and IFN- γ (□). The results are plotted as specific binding relative to an assay with no added competitor. In *b*, ME-180 monolayer cells were preincubated overnight in the absence (---) and presence (—) of $1 \mu\text{g ml}^{-1}$ of IFN- γ , then washed twice and examined for TNF- α binding as described for Fig. 2*a*. Inset, a Scatchard plot of the data.

reported previously that a human cervical carcinoma cell line, ME-180, is a very sensitive target for TNF², and we therefore used this cell line to study TNF binding in all experiments. All binding assays were carried out using a 0.5-ml total incubation volume layered on 0.2×10^6 cells plated as a monolayer. Binding was quite reproducible, each preparation of labelled TNF- α giving essentially the same results when tested in standard conditions over several weeks.

Figure 2*a* shows the time course of binding at 4, 23 and 37 °C. An initial increase in the rate of ^{125}I -TNF- α binding was observed with increase in temperature, reaching a plateau after 1 h, 2 h and 3 h at 37, 23 and 4 °C, respectively. The total binding was almost the same at all three temperatures. All subsequent experiments were carried out at 37 °C, the temperature of the cell cultures. The binding of ^{125}I -TNF- α to the cells was inhibited by various concentrations of unlabelled TNF- α ; Scatchard analysis of these data gave an apparent dissociation constant of 0.2 nM (Fig. 2*b*). The average number of binding sites per cell was $\sim 2,000$. These observations are consistent with the binding of ^{125}I -TNF- α to cellular receptors, although the identification of a specific receptor would require its characterization as a macromolecular complex with TNF and its purification.

Specificity of binding was examined by competition with highly purified recombinant preparations of TNF- β and IFN- γ . The competition with unlabelled TNF- β could be superimposed

on that obtained with TNF- α , whereas IFN- γ did not compete even at 1,000-fold excess concentration (Fig. 3a). It seems, therefore, that both TNF- α and - β compete with 125 I-TNF- α for binding to the same receptors.

It has been reported that IFN- γ is synergistic with TNF for anti-tumour action on many cell lines⁶⁻⁸. To understand the mechanism of this synergism, we pretreated the cells overnight with 1 μ g ml⁻¹ of IFN- γ and compared 125 I-TNF- α binding with untreated cells in a competition assay. Figure 3b shows the competition curve with IFN- γ -treated cells in relation to untreated cells in similar conditions. Scatchard analysis of the two curves indicated no significant change in the affinity constants, but a two to threefold increase in the number of receptors per cell was noted with interferon-treated cells. The increase in receptor number was observed consistently in four sets of experiments. Pretreatment of cells with 1 μ g ml⁻¹ of IFN- α was less effective than IFN- γ in inducing TNF receptors (data not shown).

Our experiments measured TNF binding at 37 °C. At this temperature, TNF may interact with cellular receptors in a complex way, possibly as a result of a series of metabolic events. This is suggested by our observation that the binding of 125 I-TNF after 2 h at 37 °C could not be reversed by either trypsin or salt and pH elution. However, a partial reversal (<30%) in binding was observed after 15 min (data not shown). At 4 °C, two-thirds of the total binding of 125 I-TNF could be reversed after 15 min. A similar kind of irreversible binding to that described here has been reported previously for receptors for platelet-derived growth factor¹¹. Furthermore, various characteristics of TNF binding reported here are very similar to those described for other polypeptide hormones including interferons¹¹⁻¹³. This includes a single class of specific high-affinity binding sites possessing a limited number of receptors per cell.

The inhibition of TNF- α binding by TNF- β may explain several common biological effects *in vitro* and *in vivo* observed for the two molecules^{1-8,14}. Our experiments suggest that the receptor binding domains of TNF- α and - β should be similar. Two particularly conserved regions occur at amino acids 11-20 and 121-126 (TNF- α numbering) where all residues are homologous to TNF- β . It is possible that these regions of both proteins are involved in interaction with receptors. IFN- γ is known to exhibit synergistic biological effects with several other lymphokines including IFN- α , IFN- β , interleukin-2, TNF- α and - β (refs 7, 8, 15, 16). The mechanism of such synergistic action is unknown. This is the first report of an IFN- γ -induced increase in the total number of TNF receptors. It also suggests that the receptors are central to the action of TNF. Studies are now in progress to characterize TNF receptors further and to elucidate various biochemical events which follow receptor binding and precede cell death.

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Selective immortalization of murine macrophages from fresh bone marrow by a *raf/myc* recombinant murine retrovirus

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Myeloid precursors can be grown *in vitro* in the presence of specific growth factors; however, their expansion is limited by a competing process of terminal differentiation^{1,2}. Proto-oncogenes seem to be involved in cellular proliferation and/or differentiation and may also play a role in the myelopoietic process^{3,4}. Murine myeloid precursors which are grown *in vitro* with growth factors respond with augmented self-renewal upon infection with recombinant retroviruses carrying the *v-myc* or *v-src* oncogenes⁵⁻¹¹, suggesting a synergism or complementation between some viral oncogenes (*v-onc*) and certain growth factors. We now show that the combination of two *v-onc* genes (*raf* and *myc*) induces the selective proliferation of monocytic cells from fresh murine bone marrow (BM) in the absence of a specific growth factor supplement. Depending on the culture conditions these cells can either differentiate and cease to proliferate or grow continuously, thus mimicking the alternative pathways that can be followed by committed BM stem cells *in vivo*.

Mononuclear BM cells obtained from femurs of C3H/HeJ mice were cultured in standard medium after overnight infection with retroviruses carrying *v-raf* and/or *v-myc* oncogenes. Untreated BM cells as well as cells infected with helper virus (Fig. 1a) or with retroviruses carrying only *v-raf* (3611, J1) or *v-myc* (J3, J5) (data not shown) died after a few days of culture. In contrast, in the cultures infected with the virus carrying both *v-raf* and *v-myc* (J2), clusters of non-adherent proliferating cells (J2-BM cells) could be detected 7-10 days after infection. J2-BM cells reached a peak in cell number and rate of proliferation 14 days after infection (Fig. 1a, b). At the same time, we observed a peak in frequency of colony-forming cells in soft agar (Fig. 1c). The decrease in cellular proliferation was not caused by lack of viral replication in J2-BM cells, since the fraction of virus-positive cells, as determined by infectious centre assay¹², was constant for at least 35 days following infection (Fig. 1d).

J2-BM cells growing in suspensions 14 days after infection (day 14 J2-BM cells) were characterized. Morphological examination of Giesma-stained cells revealed a relatively homogeneous cell population (Fig. 2a). The clonal expansion of day 14 J2-BM cells was further indicated by Southern blot analysis of J2-virus integration sites. The presence of a single virus-cell junction fragment (data not shown) in the DNA from the infected cultures strongly suggests that J2-BM cells are of clonal origin.

The monocytic nature of J2-BM cells was established by cytochemical and functional markers. J2-BM cells expressed nonspecific esterase, were phagocytic (Fig. 2b), and produced lysozyme ($\geq 8 \mu$ g per 10^5 cells per 72 h). No peroxidase activity was detected. Flow cytometry analysis confirmed that J2-BM cells belong to the monocytic lineage (Table 1), since more than 95% were MAC-1-positive and 85% expressed Fc receptors. A minimum of 50% of the cells expressed the mouse heat-stable antigen, asialo-GM₁ glycolipid and F4/80 antigen. In contrast, day 14 J2-BM cells were consistently negative for T-cell (Ly 1.1, Lyt 2.1, L3T4) and B-cell (surface immunoglobulins) markers.

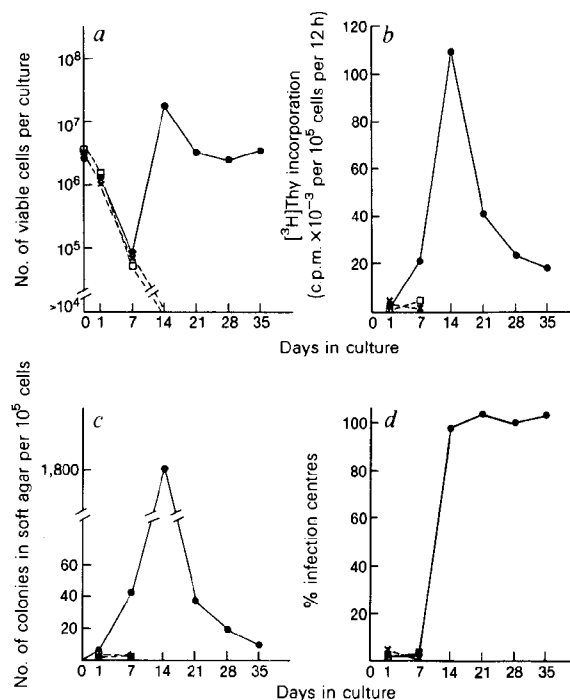
Day 14 J2-BM cells expressed helper and transforming virus-specific functions, being highly positive for production of trans-

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Fig. 1 Kinetics of cell growth, ^3H -thymidine (^3H -Thy) incorporation, formation of colonies in soft agar, and % infectious centres in BM cells after infection. A leukaemogenic strain of Moloney murine leukaemia virus was used as helper virus. The results are from representative experiments. Cell growth (a), ^3H -Thy incorporation (b), formation of colonies in soft agar (c), and % infectious centres (d) were monitored weekly in uninfected controls ($\square$), and in BM cells infected with J2 virus plus helper virus ($\bullet$) or with helper virus alone ($\times$). Each point in b and c represents the mean of triplicate samples. The standard errors were less than 5% of the mean value.

Methods. The BM cells used in these studies were obtained from femurs of C3H/HeJ mice. This strain was used as a source of BM cells because their macrophages do not respond to endotoxins which often contaminate tissue culture media and stimulate endogenous production of growth factors in BM cultures¹⁹. 5×10^6 fresh BM cells, obtained by density separation on Ficoll²⁰, were resuspended in standard medium [Dulbecco's modified Eagle's medium supplemented with penicillin (100 units ml^{-1}), streptomycin (100 $\mu\text{g ml}^{-1}$), glutamine (4 mM) and 10% fetal bovine serum] and incubated overnight in the presence of control supernatants or of viruses containing supernatants. The viruses used were the following: (1) 3611 Moloney sarcoma virus³⁰ (containing *v-raf*); (2) J1 containing a complete *raf/mil* hybrid oncogene and the 5' half of MH2 *v-myc*^{31,32}; (3) J2 (which contains a complete *raf/mil* hybrid oncogene and a complete *myc* gene consisting of the 5' half of the MH2 *v-myc* and the 3' half of MC29 *v-myc*^{30,31}); (4) J3 (derived from J2 by a 200-base pair deletion which takes the *raf/mil* gene out of the reading frame, *v-myc* remaining functional)³²; (5) J5 (which expresses a *v-myc* gene derived entirely from MC29)³².

The infections were performed at a viral concentration ranging from 1 to 5×10^4 particles per ml, as determined by fluorescence focus induction assay with anti-*v-myc*-antibodies³¹ in the case of *v-myc* carrying retroviruses J3 and J5, or by focus-forming assays in the case of *v-raf*-carrying viruses 3611 and J1. ^3H -Thy incorporation was determined in 10^5 cells pulsed with $10 \mu\text{Ci ml}^{-1}$ of [^3H]Thy (NEN) for 12 h at 37 °C. The cultures in soft agar were performed as described previously¹⁹ and the colonies (>50 cells) were scored after 10 days in culture. The infectious centres of mitomycin-treated BM cells were determined on 3T3 fibroblast indicator cells¹².



forming virus (Fig. 1d), for envelope glycoprotein gp70 (Table 1) and for *v-myc*- and *v-raf*-derived proteins. More than 90% of J2-BM cells, in fact, showed a specific nuclear staining (Fig. 2c) with anti-*v-myc* antibodies³¹ and a specific cytoplasmic fluorescence (Fig. 2d) with anti-*v-raf* antibodies¹⁴. Because of the cross-reactivity of the anti-*v-myc* or anti-*v-raf* antibodies with cellular *myc* or *raf* proteins, J2-BM cells were tested for expression of viral and cellular messenger RNA by Northern blot analysis and compared with normal or J2-virus-transformed NIH 3T3 fibroblasts. An MC29 *v-myc* DNA probe detected high levels of *v-myc* mRNA in both J2-BM cells and transformed

fibroblasts (Fig. 3a). Similarly, high expression of *v-raf* was detected using a cross-reacting human *c-raf* probe (Fig. 3b). When the blot was hybridized with a murine *c-myc* probe, which under these conditions does not recognize *v-myc*, no expression of *c-myc* was detected in J2-virus-transformed fibroblasts or J2-BM cells (Fig. 3c). These results show that J2-BM cells actively synthesized large amounts of viral *myc* and *raf* mRNA and that *c-myc* mRNA was undetectable. Therefore, the *myc* protein detected by immunofluorescence is probably of viral origin.

Infections of BM cells with J2 virus have consistently resulted

Table 1 Cell-surface markers of J2-BM cells (day 14)

Surface marker*	% Positive J2-BM cells†	Distribution
MAC-1 (M1/70)	96.8 (451)	Macrophages, granulocytes ²⁰
Fc receptor (2.4G2)	85.7‡ (284)	Macrophages, B cells ²¹
Heat-stable antigen on mouse blood cells (M1/69)	64.5§ (284)	B cells, macrophages, red blood cells, thymocytes, BM cells ²²
Asialo-GM ₁ glycolipid (anti-asGM ₁)	45.0§ (152)	Macrophages, natural killer cells, fetal thymocytes ²³
F4/80 (F4/80)	44.6§ (149)	Mononuclear phagocytes ²⁴
Ly 1.1 (39/3)	2.8 (101)	Pan-T cells, some B cells ²⁵
Lyt 2.1 (clone 49-31.1)	0.4 (93)	T-cell subsets, thymocytes ²⁶
L3T4 (H129. 19)	3.2 (105)	T-cell subsets, thymocytes ²⁷
Surface immunoglobulin (fluoresceinated goat anti-mouse [GAM]Ig)	0.7 (105)	B cells
Surface glycoprotein gp70 (37594)	94.6 (429)	Viral protein on mammalian cells ²⁸

Cells (10^6 in 50 μl) were incubated for 40 min at 4 °C with 10–50 μl of appropriately diluted antibodies or with control supernatants. They were then washed three times and incubated for 40 min at 4 °C with 10 μl of appropriately diluted fluoresceinated anti-immunoglobulins (Cappel Laboratories). After another three washes the cells were analysed for immunofluorescence on a Cytofluorograf System 30-H with a 2150 computer (Ortho Diagnostic) capable of simultaneous measurement of forward and right-angle light scatter (488 nm), red (>600 nm) and green (530 nm) fluorescence. Cells were illuminated by a 4-W argon laser (Lexel) emitting 500 MW of 488-nm light. Cells analysed for immunofluorescence were selected by forward and right-angle light scatter. Dead cells less than 1% were identified by the uptake of propidium iodine (red fluorescence) and excluded from green fluorescence analysis. A green fluorescence histogram of 1,000-channel resolution was collected from 10,000 viable cells for each sample analysed. Both unstained cells and cells treated with fluorescent second antibodies were used as negative controls for these experiments.

* The designations of the antibodies used are indicated in parentheses.

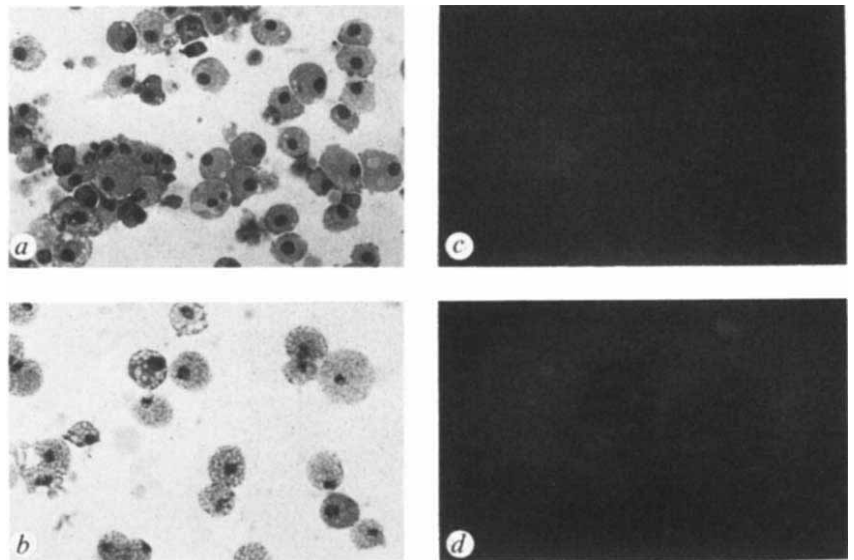
† In parentheses are shown the relative mean fluorescence units measured on ~1.5 decode log scale with 1,000 channels of fluorescence.

‡ The high percentage of Fc receptor-bearing cells was also confirmed by enumeration of rosette-forming cells²¹ with IgG-coated red cells (data not shown).

§ These percentages represent the mean number of positive cells from three experiments. Although the net calculation of positive cells is ~50%, greater than 90% of the stained cells expressed a fluorescence intensity above the mean fluorescence of the negative controls.

Fig. 2 Photomicrographs of J2-BM cells. *a*, Morphology of Giesma-stained J2-BM cells; *b*, phagocytosis of opsonized zymosan by J2-BM cells 14 days after infection with J2 virus. *c*, *d*, Specific staining of *myc*- and *raf*-derived proteins in J2-BM cells 14 days after infection. Note nuclear fluorescence (*c*) and cytoplasmic fluorescence (*d*) in cells stained with anti-*myc* serum¹³ or anti-*raf* serum¹⁴, respectively.

Methods. For *a* and *b* 10^6 cells from a 2-week-old culture were incubated for 16 h in standard medium or in the presence of zymosan ($50 \mu\text{g ml}^{-1}$) opsonized with normal mouse serum. The cells were then washed and cytopsin preparations were stained with Giesma. Cells which had ingested more than three zymosan particles were considered positive. For *c* and *d*, cytopsin preparations of J2-BM cells were fixed in acetone for 10 min, stained for 40 min with anti-*myc* (provided by Dr Karin Moelling) or anti-*raf* sera, washed three times in phosphate-buffered saline (PBS), stained for an additional 40 min with appropriately diluted fluorescein-conjugated anti-rabbit serum (Cappel) and washed three times. Less than 1% of fresh BM cells showed very weak reactivity with either antibody, whereas J2-transformed fibroblasts displayed a pattern and intensity of fluorescence similar to that of J2-BM cells (data not shown). Slides were examined and photographed at 400- and 1,000-fold magnifications using a Zeiss microscope (made available by Dr U. Saffiotti).



in selective growth of J2-BM cells at day 14. Following the initial proliferative phase associated with the presence of free floating clusters of cells (Fig. 4a), J2-BM cells formed an adherent monolayer (Fig. 4b) and ceased to proliferate. Adherent J2-BM cells were still virus producers, expressed *raf*- and *myc*-derived proteins and were morphologically indistinguishable from normal BM-derived macrophages (data not shown). Therefore, it appears that the proliferative stimulus of the J2 virus, which caused the *in vitro* expansion of the monocytic cells, is limited by a dominant differentiation process.

These considerations raised the question as to whether the differentiation was terminal, as in normal BM-derived macrophages, or whether J2-BM cells could revert to proliferating cells. We found that differentiated J2-BM cells could be induced to proliferate again by addition of dextran-based beads (CT; Cytodex, Pharmacia) to the 35-day cultures. Following addition of the beads, in four out of five experiments performed, the cells detached from plastic, became round and loosely adherent to the beads and formed floating cultures (Fig. 4c), which have been kept in continuous proliferation for more than 8 months.

J2-BM cells cultured with beads (J2-BM-CT cells) were clonable in soft agar (with an efficiency of 30–50%), indicating that their *in vitro* growth was an intrinsic property of individual cells rather than a proliferation mediated by the trophic activity of feeder cells. The plating efficiency of the J2-BM-CT cells, higher than that of the day 14 J2-BM cells (1.8%), could be the result of a progression step in the establishment of permanent cell lines from the initially infected cultures. Eighteen double-selected clones were tested for surface markers and found to be identical to each other (data not shown) and indistinguishable from the day 14 J2-BM cells (Table 1). Furthermore, all five clones tested so far produced transforming virus, suggesting that both oncogenes were active in the cloned cell lines.

Both *v-myc* and *v-raf* genes had to be expressed to induce proliferation of BM cells in standard medium, since viruses carrying either oncogene alone did not promote BM cell growth. The expression of both *v-myc* and *v-raf* therefore might have overridden the need for specific exogenous growth factor. This is consistent with earlier observations in the avian system where it was found that viruses carrying only *v-myc* augment the response of primary BM macrophages to growth factors^{9,10,16}. Moreover, in the murine system, infection of interleukin-3 (IL-3)-responsive BM cells with virus carrying *v-raf* induced their continuous *in vitro* proliferation¹⁷, whereas infection with virus carrying *v-myc* caused abrogation of IL-3 requirement¹⁸. It is possible that *v-myc* substitutes for growth factors in the prolifer-

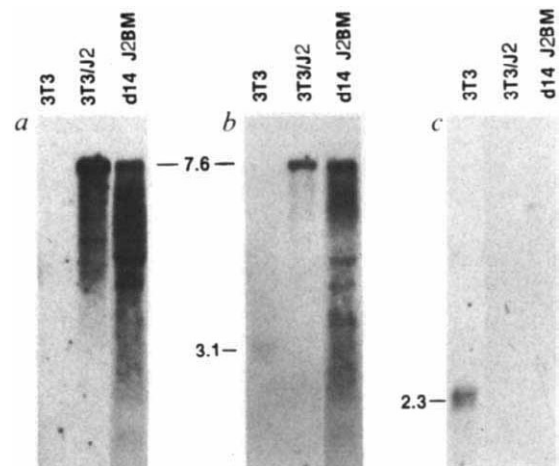


Fig. 3 Northern hybridization analysis of J2-BM cells 14 days after infection (d14 J2BM) and of J2-transformed NIH 3T3 fibroblasts (3T3/J2). Polysome-associated RNA was isolated³³ and poly(A)⁺-containing RNA selected using oligo(dT)-cellulose chromatography. This RNA (15 μg) was denatured by glyoxalation³⁴, separated electrophoretically in agarose gels, transferred to nitrocellulose and hybridized under stringent conditions to ³²P-labelled nick-translated DNA probes¹⁵. *a*, *v-myc* hybridizations. The probe was a 1.4-kilobase (kb) *Pst*I/*Aha*III *v-myc*-specific fragment of the MC29 provirus^{35,36}. The intensity of hybridization with these RNAs varied considerably; the 3T3 and 3T3/J2 samples were exposed for 1 week whereas the d14 J2BM was exposed for 20 h. *b*, *raf* hybridizations. The probe was a 2.97-kb human *c-raf*-1 cDNA³⁷. The autoradiograph was exposed for 4 days. *c*, *c-myc* hybridizations. The probe was a 5.6-kb *c-myc* *Bam*HI fragment from subclone pS107 (ref. 38). The autoradiograph was exposed for 1 week. Sizes of RNAs were determined from their migration relative to that of ³²P-labelled denatured *Hind*III fragments of bacteriophage λ . The 7.6-kb RNA is the genome-size RNA of the J2 virus³²; several smaller subgenomic RNAs are also transcribed from this provirus. The 3.1-kb transcript is the endogenous *c-raf*-1 mRNA³⁹ whereas the 2.3-kb RNA is the *c-myc* transcript⁴⁰.

ation of J2-cells. In fact, we did not detect any colony-stimulating activity in virus-free supernatants from J2-BM-CT cells, as determined by standard colony-forming assay¹⁹ with either fresh mouse BM or day 14 J2-BM cells (data not shown).

Only J2-BM cells, but not normal macrophages, could be rescued from the resting differentiated stage and reverted to

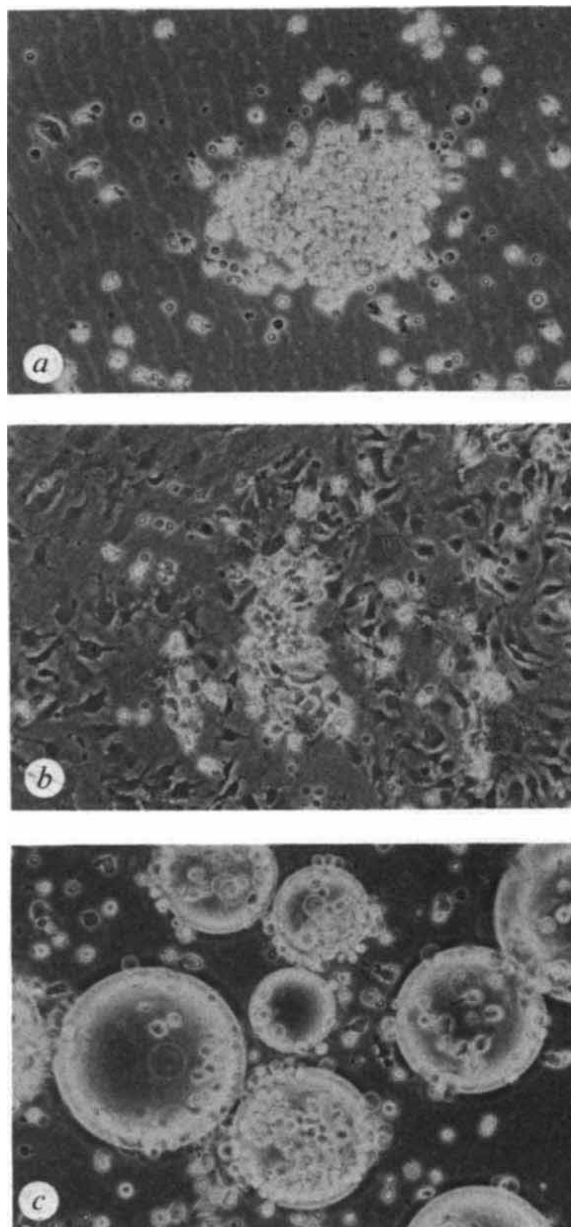


Fig. 4 Photomicrographs of J2-BM cells maintained in liquid cultures. *a*, Morphology of day 14 J2-BM cells. *b*, Morphology of J2-BM cells 35 days after infection. *c*, Morphology of J2-BM cells maintained in active proliferation by culturing them with dextran-based beads. The cells were photographed *in situ* after gently removing most of the supernatants, at 160-fold magnifications using a Zeiss microscope.

proliferating cells by co-culture with beads, suggesting that the response to the growth-promoting activity of *v-raf* and *v-myc*, down-regulated by differentiation, could be reactivated. In our system, three signals seem to be required for immortalization, defined as continuous *in vitro* growth of J2-BM cells: the two oncogenes *v-myc/v-raf* and the beads. The mechanism underlying reversal of differentiation by addition of dextran-based beads is unclear. One possibility that we are testing is the absorption of differentiating factors onto their charged surface. Identification of factors involved in the induction of differentiation could provide insights into the cellular resistance to the transforming activity of oncogenes.

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Domain structure of human glucocorticoid receptor and its relationship to the *v-erb-A* oncogene product

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The interaction of steroids with their nuclear receptors induces a cascade of regulatory events that results from the activation of specific sets of genes by the hormone/receptor complex¹. Steroids, either acting alone or possibly synergistically with other growth factors, can influence the DNA synthesis and proliferation of specific target cells, initiate developmental pathways and activate expression of the differentiated phenotype²⁻⁷. Moreover, steroid hormones have been implicated in abnormal growth regulation both in tumours and tumour-derived cell lines^{8,9}. The identification of complementary DNAs encoding the human glucocorticoid receptor (hGR) predicts two protein forms (α and β ; 777 and 742 amino acids long, respectively) which differ at their carboxy termini¹⁰. We report here that both forms of the receptor are related, with respect to their domain structure, to the *v-erb-A* oncogene product of avian erythroblastosis virus (AEV), which suggests that steroid receptor genes and the *c-erb-A* proto-oncogene are derived

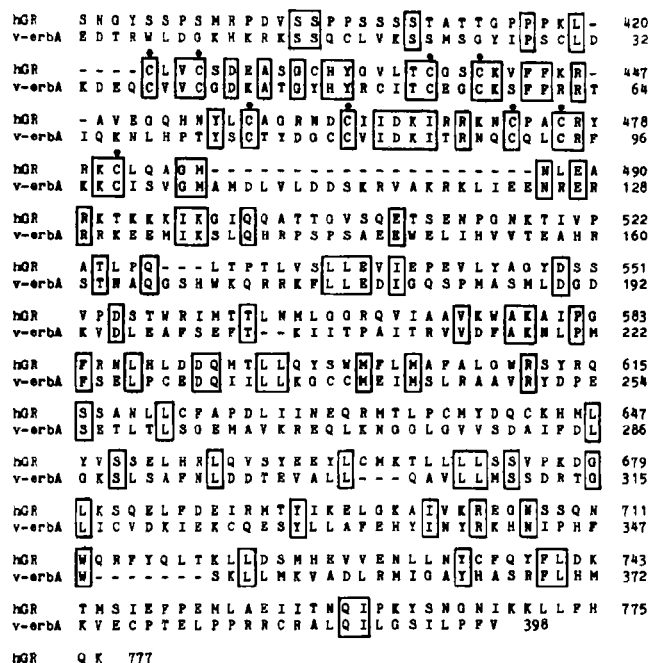


Fig. 1 Amino-acid sequence alignment of the carboxy-terminal half of the human glucocorticoid receptor (hGR) and *v-erb-A* oncogene product. Amino-acid residues 370-777 from hGR were aligned with amino-acid residues 1-398 from p75^{gag-erb-A}; identical residues are boxed and gaps are indicated by dashes. Matching cysteine residues are indicated by dots above the sequence.

from a common primordial regulatory gene. Therefore, oncogenicity by AEV may result, in part, from the inappropriate activity of a truncated steroid receptor or a related regulatory molecule encoded by *v-erb-A*. This suggests a mechanism by which *trans*-acting factors may facilitate transformation. We also identify a short region of hGR that is homologous with the *Drosophila* homoeotic proteins encoded by *Antennapedia* and *fushi tarazu*.

In the accompanying paper, we have deduced the entire amino-acid sequence of the human glucocorticoid receptor by nucleotide sequence analysis of overlapping cDNA clones¹⁰. The amino-acid sequence was scanned against a protein database maintained by R. Doolittle (University of California, San Diego) and a remarkable homology was found between the carboxy-terminal half of the receptor and the putative oncogene protein p75^{gag-erb-A} from AEV¹¹. Comparison of the last 387 amino acids of the receptor with the terminal 395 amino acids of the *gag-erb-A* fusion polypeptide revealed 85 matches, with 22% amino-acid identity overall (Fig. 1). The Needleman-Wunsch similarity value for the comparison was greater than 10 standard deviation units above the mean of 30 shuffled sequences, signifying statistically significant relatedness^{12,13}. In addition, 67 conservative amino-acid substitutions were found throughout this comparison interval. There is even greater identity (40%) between amino-acid residues 421 and 481, which corresponds to a cysteine/lysine/arginine-rich portion of the receptor. Nine out of ten cysteine residues of hGR are conserved in this region of maximal homology with *erb-A*. This is particularly significant because cysteines are not normally abundant and may participate in polypeptide tertiary structure.

In the amino-terminal half of the receptor there exist two short regions of homology with the homoeotic proteins encoded by the *Drosophila* genes *Antennapedia* (*Antp*) and *fushi tarazu* (*ftz*)^{14,15} (Fig. 2). The first region of homology (upper comparison in Fig. 2) is in the homoeo box region and the second (lower comparison in Fig. 2) precedes the homoeo box by eight amino acids. Although the match is limited, these polypeptide regions were selected from a database of 300,000 residues as the most significant matches using a comparison window of 18 amino-acid residues. The homoeo box is a 180-base pair (bp)

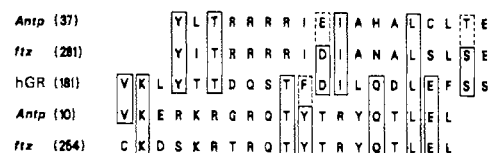


Fig. 2 Regional homology of the human glucocorticoid receptor with *Drosophila* homoeotic polypeptides. Numbers refer to the first amino-acid position in each protein segment of the *Antp* product¹⁴, the *ftz* product¹⁵, and hGR¹⁰. Broken lines indicate conservative amino-acid changes. Alignments were determined by a computer search of a database of 300,000 residues maintained by R. F. Doolittle, using an Inspect II program.



Fig. 3 Schematic representation of the primary amino-acid structure of the human glucocorticoid receptor, with the oncogene and homoeo box homologies indicated. The cysteine residues found in hGR¹⁰ are represented by arrows above the boxed coding region and basic amino acids (arginine and lysine) are denoted by solid circles. The immunogenic domain (IMM), determined from overlapping expression cDNA clones, the putative steroid-binding region (DEX) and DNA-binding domain (DNA) are indicated within the boxed receptor coding region. Numbers indicate amino-acid residues.

DNA sequence that is conserved in a variety of *Drosophila* homoeotic genes^{16,17}. These genes encode developmental regulator proteins that appear to reside in the nucleus and thus, like the glucocorticoid receptor, appear to be *trans*-acting regulatory molecules^{18,19}. Whether these aspects of steroid receptor and homoeotic protein functions are reflected in structural relatedness awaits further investigation.

Structural analysis of the glucocorticoid receptor is required for understanding the mechanisms by which it regulates gene transcription. Here we attempt to construct a model for the hGR structure based on its primary amino-acid sequence¹⁰. Previous analyses of the rat receptor molecule have identified at least three domains which are physically separable by limited proteolytic cleavage²⁰⁻²². These include a major immunogenic domain, a steroid-binding region and a DNA-binding region. The immunogenic region resides on a proteolytic cleavage fragment of relative molecular mass (M_r) ~40,000 (40K), or 45% of the estimated receptor mass²⁰; the DNA- and steroid-binding domains are found on the remaining 55% of the molecule. Additional enzymatic cleavage studies localize the steroid-binding domain on a 22K peptide fragment containing no DNA-binding activity²³.

Expression cloning techniques^{10,24} were used to isolate three distinct but overlapping glucocorticoid receptor cDNA clones. The overlap between these clones indicates that boundaries for the immunogenic region occur between amino acids 145 and 278 (Fig. 3). Localizing the immunogenic domain at the amino terminus of the receptor indicates that the DNA-binding domain may be found in the carboxy-terminal half of the molecule. The steroid-binding region may be positioned more precisely by noting the properties of the variant human β -glucocorticoid receptor¹⁰. The β -hGR form differs from the α , or steroid-binding, receptor moiety in that the 50 carboxy-terminal amino acids of the α form are substituted by 15 amino acids in the β form¹⁰. As the *in vitro* translation product encoding the β -receptor fails to bind steroid¹⁰, the steroid-binding domain is localized at the extreme carboxy terminus.

Because the Cys-Lys-Arg-rich region (amino acids 421-481) embodies a particularly striking feature of the receptor and has

regions of homology with *v-erb-A*, we speculate that this region of the carboxy terminus may embrace the DNA-binding domain. It is interesting that the *Xenopus laevis* 5S RNA gene transcription factor TFIIIA²⁵⁻²⁷ contains a cysteine-rich region composed of nine repeated domains^{28,29}. The repeats are thought to be DNA-binding structures each containing a zinc ion at its centre. Although the hGR does not contain the large number of cysteines present in TFIIIA, the structural motif of a cysteine-rich region followed by basic amino acids is maintained. Accordingly, we propose a model for the receptor as shown in Fig. 3.

The two oncogenes *erb-A* and *erb-B*, encoded by AEV, are derived from cellular genes which comprise a multi-gene family in humans³⁰⁻³². The *erb-B* oncogene is a truncated form of the epidermal growth factor receptor and transforms erythroblasts, while *erb-A* strongly potentiates the effects of *erb-B* by blocking the maturation of erythroblasts at an early stage of differentiation³³⁻³⁸. Examination of the *v-erb-A* DNA sequence reveals that its amino terminus is synthesized as a fusion polypeptide with the viral *gag* product, while its carboxy-terminal 14 amino acids are derived from a combination of viral envelope and downstream *erb-B* sequences^{11,39}. Therefore, both the amino and carboxy termini of *v-erb-A* are apparently truncated relative to the cellular homologue.

The mechanism by which *v-erb-A* potentiates transformation is unknown, but the homology between *v-erb-A* and hGR suggests that the *erb-A* genes encode a family of transcriptional regulatory molecules. Assuming that the Cys-Lys-Arg-rich region of hGR represents the DNA-binding domain, then the region retained in *v-erb-A* might also bind specific DNA sequences. Thus, *v-erb-A* could exert its transformation effects by inappropriately stimulating gene expression in erythroblasts.

These results provide evidence for the relatedness of a family of proto-oncogenes to a known transcriptional regulatory protein, the glucocorticoid receptor. hGR also shows a discrete yet significant homology to the highly conserved amino acids in the homoeo box region of the *Drosophila* homoeotic genes *Antp* and *ftz*. This region is also found in the human genome, reinforcing the suggestion that the steroid receptor is composed of several functional domains. Localization of the immunogenic region in the hGR primary amino-acid sequence and the characterization of a variant receptor which does not bind steroid have permitted us to assign structural domains using experimental data gleaned from studies of the rat glucocorticoid receptor.

The unexpected indication from this study is that the steroid receptors and the *erb-A* proto-oncogenes share a primordial archetype. By analogy with the glucocorticoid receptor, these molecules may represent a new class of *trans*-acting factors that may be candidates for enhancer sequence binding proteins. Further characterization of the relationship of the glucocorticoid receptor to these oncogene products may elucidate the evolution of a family of eukaryotic transcriptional regulatory factors.

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Cloning of the breakpoint of an X;21 translocation associated with Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder which affects approximately 1 in 3,300 males, making it the most common of the neuromuscular dystrophies (see ref. 1 for review). The biochemical basis of the disease is unknown and as yet no effective treatment is available. A small number of females are also affected with the disease, and these have been found to carry X-autosome translocations^{2,3} involving variable autosomal sites but always with a breakpoint within band Xp21 of the X chromosome (implicated by other kinds of genetic evidence as the site of the DMD lesion⁴⁻⁶). In these female patients the normal X chromosome is preferentially inactivated, which it is assumed silences their one normal DMD gene, leading to expression of the disease. In one such affected female the autosomal breakpoint lies in the middle of the short arm of chromosome 21 (ref. 2), within a cluster of ribosomal RNA genes⁷. Here we have used rRNA sequences as probes to clone the region spanning the translocation breakpoint. A sequence derived from the X-chromosomal portion of the clone detects a restriction fragment length polymorphism (RFLP) which is closely linked to the DMD gene and uncovers chromosomal deletions in some male DMD patients.

The use of rDNA sequences as probes to identify the translocation junction was complicated by two factors. First, because the human genome contains 300-400 copies of the ribosomal gene distributed in 10 clusters on the five pairs of acrocentric chromosomes, the unique junction fragment would be difficult, if not impossible, to visualize on a Southern blot of total genomic

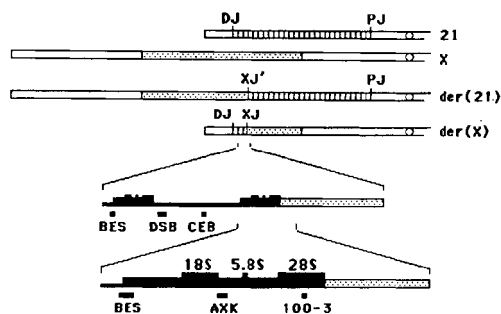


Fig. 1 Derivation of the translocation chromosomes der(X) and der(21). Below the der(X) chromosome is shown a schematic representation of the translocation chromosome at the breakpoint. The human ribosomal genes consist of a 43-kb unit repeated tandemly in blocks of 30–40 units on each of the 10 acrocentric chromosomes^{9,17}. The transcribed region is 13 kb long (represented as the solid black portion) and includes the 18S, 5.8S and 28S rRNA coding regions. The X-chromosomal portion of the translocation is represented by the stippled region, with the translocation breakpoint near the 3' end of the 28S gene. Below the schematic diagram are shown the subcloned regions BES, DSB, CEB, AXK and 100-3, used as probes.

DNA from the patient. Second, the ribosomal gene repeat unit is 43 kilobases (kb) long so that any rDNA sequence selected as a probe would have a 50% chance of being over 20 kb from the translocation junction—too far to be useful for screening Southern blots or λ libraries of 15–20-kb fragments.

To overcome the first problem, we separated the translocation chromosomes from the human acrocentric chromosomes by segregation in somatic cell hybrids generated by fusion of the patient's fibroblasts to mouse A9 cells. Three useful hybrids were generated. Hybrids A2 and F1 carried only the derivative X translocation chromosome, der(X), containing most of the X chromosome joined at band p21 to the distal end of the short arm of chromosome 21 (Fig. 1). This translocation chromosome has been shown by Southern blot analysis to carry 3–5 copies of the rDNA gene⁷. The third hybrid, C2-T10, carried the reciprocal translocation chromosome, der(21), that is, most of chromosome 21 joined at band p12 (the rDNA gene cluster) to the distal end of Xp. This chromosome was shown to carry 40–60 copies of the rDNA gene⁴. DNA from hybrid A2 [der(X)] was used to identify the junction fragment (by Southern blot hybridization) and to construct the library from which the junction fragment was isolated.

To overcome the problem of the long rDNA repeat unit, it was necessary to use as probes a series of human-specific rDNA sequences spaced over the rDNA repeat unit. Southern blot analysis of hybrid A2 cell DNA cut with a variety of restriction enzymes and hybridized with probes BES, DSB and CEB (Fig. 1) was carried out in a search for a unique restriction fragment derived from the X;21 junction region. A unique *Bam*HI fragment was revealed by the BES probe, but the presence of a similar fragment in all human DNA samples suggested that it was derived from the natural junction at the distal end of the rDNA block of chromosome 21 (DJ in Fig. 1). Further studies confirmed this notion and allowed the ribosomal gene complex to be oriented as shown in Fig. 1, with transcription in the telomere to centromere direction (R.G.W. *et al.*, manuscript in preparation). The X;21 junction was not revealed by these probes, which suggests that the junction must be near the 3' end of the 28S gene, the only region not adequately covered by the three probes. To examine that region in more detail, a 600-base pair (bp) *Xba*I–*Kpn*I fragment (AXK, Fig. 1) was isolated from the transcribed spacer region. This probe, when hybridized to *Xba*I, *Pst*I and *Hind*III digests of hybrid A2 cell DNA, revealed, in addition to the expected rDNA bands, additional unique bands at an intensity corresponding to one copy per cell⁸. This result allowed a map of the junction region to be constructed

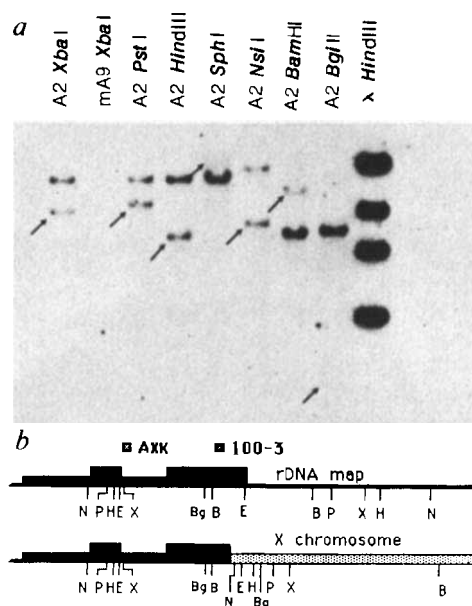


Fig. 2 Identification of the X;21 junction fragment by Southern blotting. **a**, DNAs were isolated¹⁸ from cultures of hybrid A2 and mouse A9, digested with the restriction enzymes indicated, separated by electrophoresis on a horizontal 0.6% agarose gel and transferred to nitrocellulose¹⁹. The human-specific rDNA subclone 100-3 was ³²P-radiolabelled by nick-translation¹⁹. Prehybridization of nitrocellulose filters was at 50 °C in 50% formamide, 5 × SSPE (1 × SSPE = 0.18 M NaCl, 0.01 M sodium phosphate (pH 7.4), 0.001 M EDTA), 5 × Denhardt's, 150 μ g ml⁻¹ sheared salmon sperm DNA, 0.1% SDS. Hybridization was in the same solution plus 10% dextran sulphate and 3 × 10⁶ c.p.m. ml⁻¹ of radiolabelled DNA. The filter was washed in 0.1% SDS and 0.2 × SSPE several times at room temperature and twice at 65 °C for 30 min each. Autoradiography was at –70 °C with an intensifying screen for 6 days. The arrows indicate unique human-specific bands derived from the translocation breakpoint. The major bands in each track indicate fragments derived from the 3–4 normal rDNA repeat units found on this chromosome. **b**, Restriction map of the normal rDNA repeat unit¹⁰ and the translocation junction. The map of the translocation junction was derived from Southern blot analysis of hybrid A2 DNA probed with either AXK or 100-3. The probe binding sites are indicated at the top of the figure. Restriction sites: B, *Bam*HI; N, *Nsi*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; and X, *Xba*I.

with putative *Xba*I, *Pst*I and *Hind*III sites located in X-chromosomal DNA (Fig. 2).

Using this information, numerous attempts were made to clone the translocation junction using AXK as a probe. These experiments included construction of both phage and plasmid libraries from either partial digests of hybrid DNA or size-selected DNA fragments isolated by agarose gel electrophoresis. None of these cloning attempts yielded the junction fragment, forcing the conclusion that either ribosomal sequences or X-chromosomal sequences from this region were refractory to cloning. The former possibility seemed most likely as previous experiments had suggested that clones carrying the transcribed spacer region and 28S gene were underrepresented in total genomic libraries⁷. In order to isolate a human-specific probe much closer to the translocation breakpoint, the human 28S ribosomal gene sequence¹⁰ was compared with the homologous mouse sequence¹¹. Three human-specific regions were identified, subcloned and used to probe Southern blots of genomic DNA from the hybrid cell line. The results obtained for one of these probes (100-3) are shown in Fig. 2a. In addition to the restriction fragments previously identified with AXK, several new bands were visualized, including a 12-kb *Bam*HI fragment containing the translocation breakpoint, which carried at most 1.8 kb of rDNA. To clone the junction fragment, DNA from the A2 hybrid line was cut with *Bam*HI and size-selected fragments of 11–15 kb were cloned into λ Charon 35 (ref. 12).

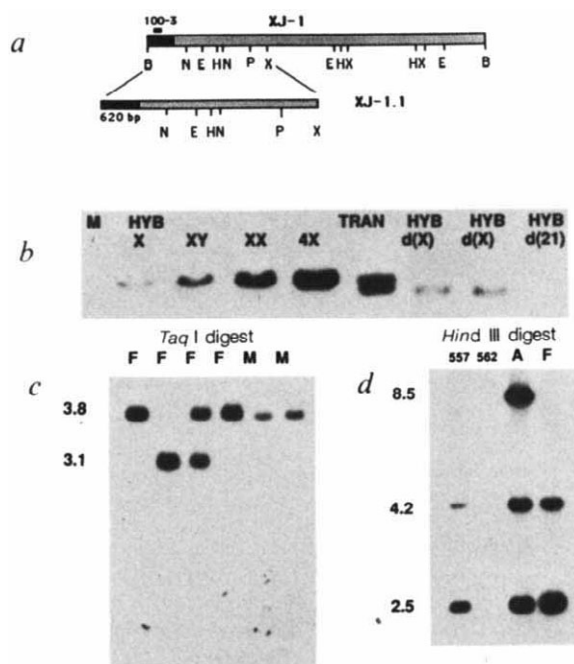


Fig. 3 *a*, Restriction map of the translocation junction clone XJ-1. The stippled area represents X-chromosomal material. The solid region represents that part derived from the rDNA cluster on chromosome 21. The translocation breakpoint is 620 bp from the 5' *Bam*HI site (unpublished data). The 100-3 probe binding site is indicated above the figure. The XJ-1.1 subclone from which the 1-kb *Nsi*I fragment was derived is indicated below the map. The restriction site designation is described in Fig. 2. *b*, Localization of XJ-1 to the X chromosome. A Southern blot of total genomic DNA from several cell lines was probed with the 1-kb *Nsi*I fragment derived from XJ-1.1. The lanes contain 5 µg of *Bam*HI-digested DNA from: M, mouse A9; HYB X, AHA 11a, an X-only mouse/human hybrid; XY, normal male; XX, normal female; 4X, a human female line with four X chromosomes; TRAN, the X;21 translocation patient; HYB d(X), the A2 and F1 hybrid lines carrying the der(X) chromosome; HYB d(21), the C2-T10 hybrid carrying the der(21) chromosome. The normal X chromosomes generate a 15-kb *Bam*HI fragment (lanes 2-5); the patient (lane 6) has a normal 15-kb band and a 12-kb translocation band; the hybrids carrying only the der(X) translocation chromosome (lanes 7, 8) have only the 12-kb band. *c*, A *Taq*I polymorphism associated with XJ-1.1. DNAs from four unrelated females (F) and two males (M) were digested with *Taq*I, electrophoresed, blotted and hybridized to the 1-kb *Nsi*I fragment from clone XJ-1.1 as described in Fig. 2 legend. The probe hybridizes to a 3.8- and a 3.1-kb fragment in females heterozygous for the RFLP (lane 3) and to either a 3.8- or a 3.1-kb band in homozygous females (lanes 1, 2 and 4) and males (lanes 5, 6). *d*, Analysis of deletions for XJ-1.1 in males with DMD. DNAs from two unrelated males with DMD (557 and 562), the translocation patient (A) and her father (F) were digested with *Hind*III and probed with the *Nsi*I fragment from XJ-1.1. Of the 50 DMD males tested in this manner, 49 had the normal banding pattern as seen with patient 557, that is, a 4.2- and a 2.5-kb band. One patient (562) showed no hybridizing sequences, indicating a deletion of this part of the chromosome. The translocation patient (A) shows a complex pattern consisting of a 4.2- and a 2.5-kb band derived from her normal X chromosome plus a 4.2- and an 8.5-kb band from her translocation chromosome. Her father (F) shows a normal banding pattern.

Approximately 10^6 recombinant phage were plated on *Escherichia coli* DB1161 (recA recBC⁻sbCB)¹³ and screened with 100-3, yielding five positive clones. Restriction mapping of these clones revealed all five to be identical. The map of the insert of one of these phage (XJ-1) is shown in Fig. 3a. The cloned fragment contained all of the restriction sites revealed by Southern blotting, consistent with its being the junction fragment. To further establish its identity as the junction fragment, a 1-kb *Nsi*I fragment, free of repeat sequences, was isolated from a *Bam*HI-*Xba*I subclone (XJ-1.1) and used to probe Southern blots of *Bam*HI-digested DNA from genomes with one, two or four human X chromosomes. The results (Fig. 3b) reveal a hybridization signal proportional to the number of human X chromosomes. This fragment also hybridized

Table 1 Linkage of XJ-1.1 to DMD

Family	Phase known		Phase unknown segregation
	Non-rec.	Rec.	
1			3:0
2			5:0
15	2	0	
17	1	0	
22			2:0
27			3:0
Total:	3	0	13

Rec., recombinant.

to a mouse-human hybrid carrying an X chromosome as the only human chromosome (Fig. 3b) and to one carrying only the short arm of the X chromosome (not shown). The hybridization pattern is also consistent with a translocation junction origin. In Fig. 3b, the *Nsi*I fragment of XJ-1.1 reveals a 15-kb *Bam*HI fragment from a normal X chromosome, a 12-kb band (that is, the cloned portion) from hybrid A2 with the der(X) translocation chromosome, and a double band from the patient's DNA, reflecting her two X chromosomes. A similar heterozygotic pattern in the patient's DNA was revealed with eight other restriction enzymes tested.

To show that the translocation junction is closely linked to the DMD locus, two studies were done. First, the *Nsi*I fragment of XJ-1.1 was used to seek RFLPs by probing Southern blots of DNA from four unrelated females cleaved with 27 restriction enzymes. A polymorphism detected with *Taq*I (Fig. 3c) was used to examine segregation patterns in several DMD families. Of 83 females in these families, 23 were heterozygous for the *Taq*I RFLP and no recombinants were found between XJ-1.1 and DMD in 16 meiotic events (Table 1). Of these 16 meiotic events there were 3 of known phase and 13 of unknown phase. All results were obtained with affected or normal sons of obligate DMD heterozygous carriers. A LOD score was calculated¹⁴ based on segregation of the *Taq*I RFLP in these DMD families and yielded a maximum of 3.4 at $\theta = 0$ centimorgans. The sample size of 16 meiotic events used in this analysis is quite small and many more informative families will be required to give a more precise estimate of linkage distance.

The second approach was to test a set of 50 DMD patients for deletion or rearrangement of the XJ-1.1 region. One of the 50 males (patient 562) displayed no hybridizing sequences (Fig. 3d), indicating deletion of the XJ-1.1 region. Recently, Kunkel *et al.*¹⁵ have isolated a probe (pERT87) closely linked to DMD which maps distal to XJ-1 and have detected deletions in 5 of 57 unselected DMD patients¹⁶. We have probed Southern blots (provided by Dr L. Kunkel) of DNA from all of these patients and found that all five also show deletions of XJ-1.1. Conversely, our panel of 50 DMD patients was screened by Kunkel for hybridization to pERT87. Only one patient, 562, failed to show hybridization to this probe (Kunkel, personal communication). The fact that these deletions are thought to be quite small (250-600 kb) suggests that XJ-1.1 and the probes described by Monaco *et al.*¹⁶ are closely linked to each other and to the DMD locus.

The underlying assumption in this work is that the translocation event disrupted the DMD locus, causing expression of the disease. Thus the junction clone or clones derived from chromosome walking should make it possible to find RNA transcripts from this region and eventually identify the gene product itself.

At a more immediate level, the translocation fragment will provide new RFLPs for use in carrier detection and prenatal diagnosis of DMD. This new RFLP will complement existing Xp21 probes and together with those described recently by Monaco *et al.*¹⁶ should provide enough closely linked markers to allow accurate diagnosis of most potential carriers.

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An intron in the 23S ribosomal RNA gene of the archaeobacterium *Desulfurococcus mobilis*

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The archaeobacteria have been defined, at a molecular level, as constituting a third primary kingdom consisting of the methanogens, the extreme halophiles, and the sulphur-dependent extreme thermophiles. In reaching this conclusion, Woese and colleagues used the 16S ribosomal RNA as an approximate chronometer for evolutionary time and demonstrated that, at a nucleotide sequence level, the archaeobacteria are as different from the eubacteria and eukaryotes as the latter kingdoms are from one another^{1,2}. Current research on archaeobacteria is yielding valuable insights into the evolutionary relationships between archaeobacteria, eubacteria and eukaryotes, and into the early forms of cellular life³. Here, we extend this knowledge by providing the first evidence for the occurrence of an intron within any prokaryotic ribosomal RNA. The intron was found within the 23S rRNA gene of the sulphur-dependent and anaerobic Thermoproteale *Desulfurococcus mobilis*, which was isolated from hot acidic springs in Iceland at temperatures up to 97 °C^{4,5}. The intron contains 622 base pairs (bp); it is very A + T-rich (65%) compared with the 23S rRNA gene (34%), and it exhibits a large open reading frame. The splicing site occurs in domain IV of the 23S RNA at a position close to that of an intron of the lower eukaryote *Physarum polycephalum*⁶; the intron does not readily fall into one of the three classes of eukaryotic nuclear introns because it has features in common with those of classes I (rRNA) and III (transfer RNA)⁷.

23S rRNA genes from two archaeobacteria, the extreme thermophile *D. mobilis* and the extreme halophile *Halococcus morrhuae*, have recently been sequenced in this laboratory, and both exhibit sequence and secondary structural features that are

AGGGCAGGGG	GTTCACCTTA	AAAATTAGCA	TTATCTTATG	TTGCATGGGG	TAGGATTAA	GCATAATAAT	GAGAAATGTA
10	20	30	40	50	60	70	80
GTGGAATATC	TOCTTACCTG	CTTGGATTGA	TAATAGGTGA	TGGAGGACTT	TACAAGTTAA	AATAAAGGG	TAACAGAAGC
90	100	110	120	130	140	150	160
GAATATAAGG	TTGTAATAAC	GCAAAAGTCT	GAAAACCTTA	TTAAACAACA	CATAGCACCA	TTAATGCAAT	TTCTCATAGA
170	180	190	200	210	220	230	240
TGAACATAAT	GTGAATCAA	AAATACAGAT	CGTTAAGGOT	GATACAAGAT	ATGAGTTAAT	AGTATCCAGT	AAGAACTAAT
250	260	270	280	290	300	310	320
ACTATTATTT	CGCTAACATG	CTAGAGAGGA	TAAGGTTATT	CAATATGCGT	GAGCAAAATG	CUTTCATAAA	GGGGCTATAT
330	340	350	360	370	380	390	400
GTAGCTGAAG	GAGATAAAAC	CCTCAAGAGA	CTAAGAATTT	GGAATAAGAA	TAAAGCATTA	CTAGAAATAG	TATCGCGGATG
410	420	430	440	450	460	470	480
GTTAATAAAC	CTGGGTGTAA	GGAATACTAT	TCACCTGGAT	GATCATAGGC	ACGGTGTATA	TGTATTAAAT	ATTTCACCTCA
490	500	510	520	530	540	550	560
GAGATAGAAT	TAAGTTTOTT	CACACAATTC	TCTCTTCACA	CCCTTAACCCC	CTGCCCCCGG	AG	
570	580	590	600	610	620		

Consensus sequence	YUCANNAGACUA	<variable>	UNANGANAUAGUC
<i>D. mobilis</i>	UCAAGAGACUA	< 20 n >	ACUAGAAUAGUA
<i>P. polycephalum</i>	CGAAGAGACUA	< 26 n >	UAAAGAGAUAGUC

Fig. 1 Nucleotide sequence of the 622-bp intron, showing the strand corresponding to the transcribed RNA. The consensus sequences that may be required for the splicing of the eukaryotic rRNA introns are underlined in the sequence, and they are aligned below it (see text). The sequence was determined as follows. A 4-kilobase *Bam*HI fragment, subcloned into M13mp18, was digested with restriction enzymes specific for 4 bp. The DNA fragments were fractionated on a 4% polyacrylamide gel and cloned into M13mp18 or mp19 in both directions. Their directions were determined by 'figure 8' mapping²⁶. Single-stranded DNA was prepared and used as a template for DNA sequencing by the dideoxynucleotide method⁸, using [α -³²S]ATP (1,000 Ci mmol⁻¹; Amersham); the DNA was run on sequencing gels containing 4-6% polyacrylamide and salt or wedge-shaped gradients²⁷. Both DNA strands are sequenced, at least twice, and overlaps were obtained at each restriction site. Some differences were observed between our restriction map of the 23S rRNA gene and that published by Neumann *et al.*⁹. Some of the restriction enzyme cuts that we found were reported by Neumann *et al.* only for the closely related *D. mucosus* rRNA operon, and our estimate of the 16S-23S RNA spacer region was larger. However, we agree that the 5S RNA and 23S RNA genes are uncoupled, which is an exclusive property of the *D. mobilis* operon⁹.

exclusive to the archaeobacteria (H. Leffers, J.K. and R.A.G., unpublished work). These sequences were aligned with those of other eubacterial and eukaryotic 23S-like rRNA genes and the *D. mobilis* gene was shown to contain a large 622-bp insert. Its nucleotide sequence is given in Fig. 1.

To establish whether the insert was, indeed, an intron, the 23S RNA was sequenced over the putative splicing site using reverse transcriptase. A 5' end-labelled restriction fragment was used as a primer with purified 23S RNA as the template; sequences were obtained using dideoxynucleotides⁸. Figure 2 shows an alignment of the resulting complementary DNA sequence with that of the 23S RNA gene. The result demonstrates that the insert was excised from the pre-RNA and thereby establishes both the nature and location of the intron.

Southern hybridization studies between the purified large rRNAs and total chromosomal DNA confirmed the result of Neumann *et al.*⁹ that only one operon exists for the large rRNAs (data not shown). There can be little doubt, therefore, that this gene is both transcribed and spliced. This view, that it is not a pseudogene, was reinforced by demonstrating that 300 nucleotides of cDNA, sequenced around the splicing site (Fig. 2) and the 5' end of the 23S RNA, were identical to the gene sequence.

The intron is located within the functionally important domain IV of the 23S-like RNAs¹⁰ (Fig. 3). The sequence shown is that of *D. mobilis*, while the numbering system and secondary structure are based on *Escherichia coli* 23S RNA¹¹. The uncertainty in the assignment of the splicing site, at nucleotide 1,953 ($\pm$ 1), is due to an A-G direct repeat occurring at the exon-intron junctions. The insert is flanked by highly conserved sequences that contain several post-transcriptionally modified nucleotides in *E. coli*¹⁰ (Fig. 3) and at least one in *D. mobilis* 23S RNA

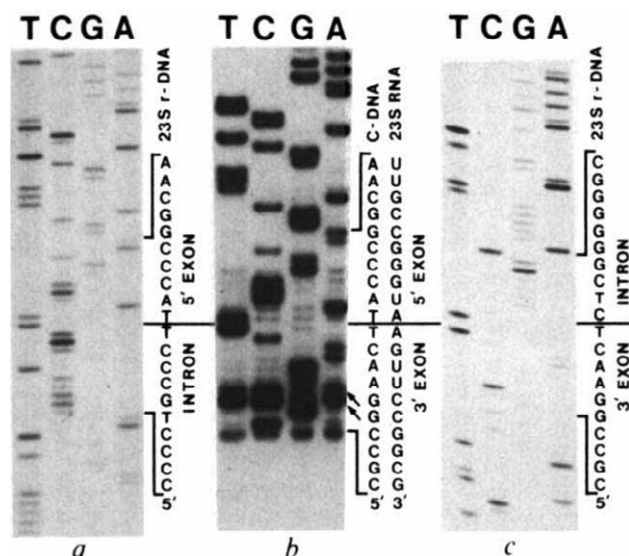


Fig. 2 Gel *b*, sequencing gel showing the sequence of cDNA obtained through the splicing site using reverse transcriptase. This gel is flanked by DNA sequencing gels showing the 5' (*a*) and 3' exon-intron junctions (*c*). Over 200 nucleotides of the cDNA sequence were read and they corresponded exactly to the gene sequence.

Methods. The gene sequence was determined by hybridizing 3 pmol of a double-stranded *Hha*I-*Hae*III restriction fragment, which had been 5' end-labelled using [γ - 32 P]ATP (6,000 Ci mmol $^{-1}$; ICN Pharmaceuticals), to 5 pmol 23S RNA. The hybridization reaction was performed in 15 μ l buffer (20 mM HEPES pH 7.0, 0.4 M NaCl, 1 mM EDTA, 89% formamide) by heating at 95 °C for 2 min, then at 55 °C for 90 min, and cooling quickly. Sequencing reactions were performed in 10 μ l buffer (50 mM Tris-HCl pH 8.3, 10 mM MgCl $_2$ and 2 mM dithiothreitol) containing 200 μ M dNTP, 200 μ M of the appropriate dideoxy dNTP, 0.5 pmol primer template complex and 10 units AMV reverse transcriptase (Life Sciences, Florida). cDNA was fractionated on 6% polyacrylamide gels (20 cm $\times$ 40 cm $\times$ 0.02 cm). Strong termination of nonspecific nature was detected in the cDNA track at G 1,960; this may result from a modification of G 1,959.

(Fig. 2). Chemical modification studies have also indicated that part of this region is accessible on ribosomes of both eubacteria and eukaryotes¹¹. Each of these characteristics (sequence conservation, post transcriptional modification and location on the ribosomal surface) is a strong indicator of a functionally important region of RNA, although no definite function has yet been established¹⁰.

It appears to be a general property of rRNA (and tRNA) introns that they are clustered in functionally important regions. Two rRNA introns have been located in neighbouring positions of domain IV: one occurs in the *P. polycephalum* gene at position 1,949, which is 3–5 nucleotides 5' of the present intron⁶, and another occurs in *Tetrahymena pigmentosa* at position 1,925 (ref. 12) (Fig. 3). Most of the others have been located in a region of domain V that has been strongly implicated in the peptidyl transfer reaction¹¹. The function(s) of the introns remains unclear. The splicing reaction could be a mechanism for activating ribosomes after assembly¹³; it could also constitute a proofreading for the correct assembly of important functional sites.

Eukaryotic nuclear introns have been divided into three groups on the basis of their splicing mechanisms: (I) rRNA; (II) mRNA and (III) tRNA⁷. In order to classify the present intron, we aligned its sequence with the 501-nucleotide intron that occurs at an adjacent position in the 23S-like rDNA of *P. polycephalum*⁶. Two common sequences were detected at similar relative positions (Fig. 1). They correspond to the consensus sequences of class I introns that are essential for correct

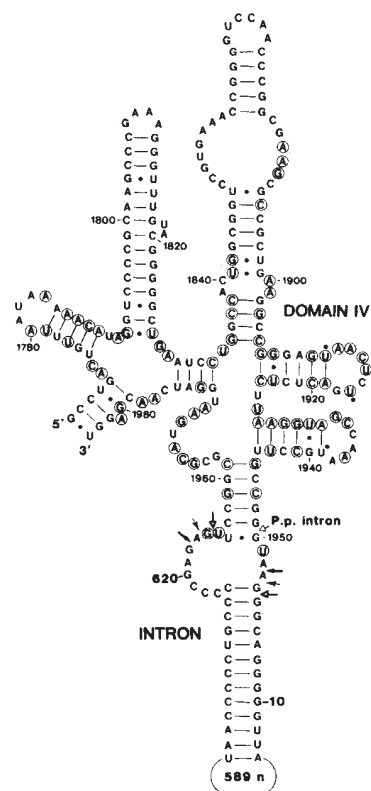


Fig. 3 Secondary structure of a major part of domain IV of the pre-23S RNA of *D. mobilis*. The numbering system and the secondary structure derive from that of *E. coli* 23S RNA¹¹. The ringed nucleotides are highly conserved in 12 sequenced 23S-like RNAs from eubacteria, archaebacteria, chloroplasts and the eukaryotic cytoplasm¹⁰. Domain IV of *E. coli* 23S RNA¹¹ contains several modified nucleotides and these are indicated by asterisks. *D. mobilis* exhibits at least one modification, at G 1,959 (see Fig. 2). The three pairs of arrows indicate the three possible splicing junctions, and the putative helix at the start of the intron is shown. P.p. intron, the position of the *P. polycephalum* intron 2 (ref. 6).

splicing^{14–16}. Other properties, however, are not shared by class I introns; these include the putative base pairing between the ends of the RNA (Fig. 3), the lack of internal core structure, and the absence of a uridine at the 3' end of the 5' exon^{14,15,17}. The latter are characteristics of class III introns that occur in the anticodon loops of nuclear tRNAs; these exhibit no obvious consensus sequences and the splicing reaction is probably directed by an endonuclease recognizing invariant features of the tRNA structure^{18,19}.

Large open reading frames (ORFs) are common to the transcribed introns of many mitochondrial mRNAs, some large rRNAs and chloroplast tRNAs^{15,20}. As illustrated in Fig. 4, the intron in *D. mobilis* also contains one ORF that starts 57 ($\pm$ 1) nucleotides from the 5' exon-intron junction, continues through



Fig. 4 The locations of stop codons in the three reading frames of the RNA that is transcribed from the intron and the flanking region of the 23S RNA gene. ORF denotes the open reading frame. The plot was obtained using a Staden computer program²⁸.

the 3' junction and extends 215 (± 1) nucleotides into the 3' exon. The likelihood that it codes for a protein is reinforced by the fact that the other frames of the intron contain 28 and 30 stop codons. Moreover, two putative Shine-Dalgarno sequences (5' AGGA 3' and 5' GGGGT 3') precede the ORF; these lie 3 and 7 nucleotides, respectively, from the start codon and both can interact with the sequence 5' ACCTCCT 3' at the 3' end of the 16S RNA (data not shown). Furthermore, a 30-nucleotide region preceding these putative Shine-Dalgarno sequences is very A + T-rich (80%); this feature is common to ORFs of introns in the rRNA genes of *Neurospora crassa*²¹. The extension of the ORF by 215 (± 1) nucleotides into the 3' exon suggests either that it is translated prior to splicing or that the RNA circularizes after splicing, as was found for intron II of *T. pigmentosa*²², such that translation is terminated by stop codons upstream from the initiation codon.

In conclusion, this is the first intron to be found in a rRNA gene of any prokaryotic system. The result complements those introns found in archaeobacterial tRNAs, that is, the small putative introns in tRNA^{Leu} and tRNA^{Ser} of *Sulfolobus solfataricus*²³ and a 105-bp intron in the tRNA^{Trp} gene of *Halobacterium volcanii*²⁴; all exhibit important differences compared with their eukaryotic counterparts. Collectively, though, these results reinforce the view that the archaeobacteria, in general, share more characteristics with the eukaryotes than do the eubacteria, where an intron has been reported only for the thymidylate synthase gene of the phage T4²⁵.

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Near-reciprocal phenotypes caused by inactivation or indiscriminate expression of the *Drosophila* segmentation gene *ftz*

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Early in development, *Drosophila* embryos express the segmentation gene *fushi tarazu* (*ftz*)^{1,2} in a 'zebra' pattern of active and inactive stripes, each about the width of a segment primordium³. If the *ftz* gene is prevented from functioning, alternating portions of the body normally derived from the active stripes fail to develop, resulting in larvae which lack the denticle bands normally formed by the mesothorax and odd-numbered abdominal segments (that is, thoracic segment T2 and abdominal segments A1, A3, A5 and A7). Here, using the *Drosophila* heat shock protein 70 (*hsp70*) gene promoter⁴⁻⁷ to drive widespread expression of *ftz* transcripts on heat shock, I find that unrestricted *ftz* activity can cause a reciprocal 'pair-rule' phenotype—that is, the absence of the denticle bands which are normally derived from segments T1, T3, A2, A4, A6 and A8. These results show that both the 'on' and 'off' states of *ftz* gene expression have instructive roles in the development of alternating regions of the body, and hence suggest that the *ftz* gene acts combinatorially with other pair-rule genes (for example, *even-skipped*, *odd-skipped*, *paired*)⁸⁻¹⁰ to establish the metameric pattern of the body.

To examine the consequences of indiscriminate *ftz* expression, a sequence coding for most of the mature *ftz* messenger RNA (including the entire open reading frame of the *ftz* protein)¹¹ was fused to the promoter of the *hsp70* gene⁵⁻⁷, and this hybrid gene was incorporated into the germ line by P-element-mediated transformation^{12,13} (see Fig. 1). Four independent transformant lines were obtained: two, called HSF2 and HSF3, have been examined in detail. The HSF2 and HSF3 transformants result from insertions of a single copy of the *P(hsp70-ftz, Adh⁺)*

element into chromosomes 2 and 3, respectively (segregation and Southern blotting data not shown): in both cases, animals homozygous for the transduced element are viable and fertile.

The *hsp70* promoter can be induced by heat shock throughout the life cycle except for the first few hours of embryogenesis (up to the blastoderm stage, ~2.5-3.5 h after egg laying, 25 °C) and the late stages of oogenesis^{5-7,14}. Both HSF transformants expressed the *hsp70-ftz* hybrid transcript in response to heat shock during the blastoderm stage as well as in older embryos (see, for example, Fig. 2). When HSF embryos were heat-shocked during the blastoderm stage, approximately half of the resulting larvae displayed pair-rule phenotypes (described below and in Fig. 3). Similar segmentation phenotypes were not observed when HSF embryos were exposed to heat shock after the blastoderm stage, nor were they observed when control embryos lacking the *hsp70-ftz* hybrid gene were heat-shocked at any time during embryogenesis.

Previous studies have shown that almost all cells of the body induce the *hsp70* promoter on heat shock⁵⁻⁷. Because wild-type embryos normally display a tightly restricted zebra pattern of *ftz* transcripts during the blastoderm stage, the altered segment patterns caused by heat-shocking HSF embryos during this period probably result from ectopic expression of the *hsp70-ftz* transcripts.

Before describing the HSF phenotypes, it is helpful to consider the pair-rule phenotype caused by apparent null mutations in the *ftz* gene. *ftz*⁻ embryos develop into larvae which appear to delete every other segment^{1,2} (that is, segments T2, A1, A3, A5 and A7; see Fig. 3b, c). This assessment is based primarily on the presence or absence of conspicuous belts of ventral hairs (denticle bands) which are formed by anterior portions of each segment. However, examination of sensory organs such as Keilin's organs, which are positioned at or near the anteroposterior compartment boundary¹⁵, as well as the patterns of dorsal hairs, which display segment-specific differences in both anterior and posterior compartments of several segments^{15,16}, indicates that the deleted regions are composed of segment-length domains which begin and end near the anteroposterior compartment boundaries within adjacent segments (Fig. 3; see also refs 9, 10). Note that the regions deleted

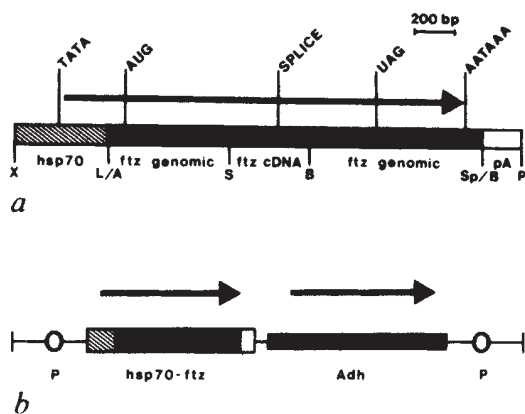


Fig. 1 Composition of the *hsp70-ftz* fusion gene (a) and generation of germline transformants (b). a, The fusion gene is composed of three pieces: (1) a 450-base pair (bp) sequence containing the *hsp70* promoter (hatched bar), (2) a composite *ftz* gene which contains the uninterrupted open reading frame present in the mature *ftz* transcript (solid bar), and (3) a 150-bp fragment of the 3' end of a *Xenopus* β -globin complementary DNA including a 23-bp poly(A) tail (open bar). The *hsp70* (132E3) and globin fragments are described in refs 33 and 34; the composite *ftz* gene was generated by substituting a 580-bp region of the genomic clone *Dm437* (ref. 35) containing the single *ftz* intervening sequence, with the corresponding 400-bp region of the cDNA clone *G20*⁺ (ref. 11) as shown (X, A, S, B, Sp and P represent, respectively, the *Xba*I, *Ava*II, *Sal*I, *Bgl*II, *Sph*I and *Pst*I sites; / indicates that the fragments are joined by blunt-end ligation) (see ref. 11 for the *ftz* cDNA and genomic sequences). The 5' portion of the fusion gene transcript (arrow) consists of 200 bp of the 5'-untranslated *hsp70* transcript joined by a 10-bp linker sequence (L = AAGCTTGGGC) to the *ftz* gene about 80 bp in front of the start of the major open reading frame (AUG). The 3' end of the fusion gene, beginning at the end of the open reading frame (UAG), consists of ~400 bp of the *ftz* 3'-untranslated region, the putative polyadenylation signal (AATAAA), and the next 100 bp of the *ftz* genomic sequence. b, The *hsp70-ftz* fusion gene was inserted into the P-element transformation vector pPA-1 (J. Posakony, unpublished) which carries the *Adh* (alcohol dehydrogenase) gene as a selectable marker. The directions of transcription of the *hsp70-ftz* and *Adh* genes are indicated by arrows; P, sites for P-factor-mediated integration. Germline transformants were then generated in *Adh*^{fr} *cn*; *ry* hosts by standard means^{12,13}. The same hosts were used as untransformed controls for the heat-shock experiments.

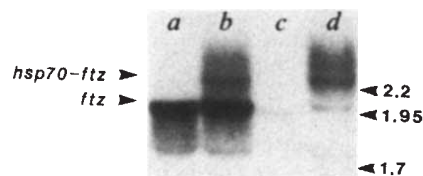


Fig. 2 Heat-shock control of the *hsp70-ftz* fusion transcript. The presence of normal *ftz* and hybrid *hsp70-ftz* transcripts was assayed by Northern blot analysis using a probe complementary to the composite *ftz* gene (solid bar in Fig. 1a). a, HSF2 embryos aged 2 3/4–3 1/4 h after egg laying express the normal *ftz* transcript (~2.0 kilobases (kb))^{11,35,36} and, at a low concentration, the 2.2-kb hybrid *hsp70-ftz* transcript (not visible in this exposure). b, HSF2 embryos of a similar age to those in a, but heat-shocked for 20 min, express the 2.0-kb *ftz* transcript as well as a series of larger transcripts, including a discrete 2.2-kb transcript: the 2.2-kb transcript almost certainly corresponds to the *hsp70-ftz* transcript (which should be ~200 bp longer than the normal *ftz* transcript if terminated near the putative polyadenylation signal), and the larger transcripts may correspond to hybrid transcripts which terminate farther downstream. Note that the *hsp70-ftz* transcripts are less abundant than the native *ftz* transcripts; this difference may be due to poor induction of the fusion gene in some embryos (consistent with the finding that only about half of the HSF embryos heat-shocked during this period give rise to larvae showing reciprocal *ftz* phenotypes). It may also indicate that the levels of ectopic *hsp70-ftz* transcripts necessary to alter the segment pattern may be lower than the levels of *ftz* transcripts normally expressed in the zebra pattern. c, Control embryos (6–10 h after egg laying) heat-shocked for 20 min express low levels of the normal *ftz* transcript. d, HSF2 embryos of a similar age to those in c, but heat-shocked, express low levels of the normal *ftz* transcript as well as high levels of the fusion transcripts.

Methods. Embryos were grown at 25 °C and heat-shocked at 35 °C for 20 min. Northern blot analysis was performed as described previously³⁷ (~8–10 μ g of total nucleic acid was loaded per lane). The *ftz* probe was generated by gel extracting and then nick-translating of the composite *ftz* gene. Neighbouring lanes loaded with RNA from 2 3/4–3 1/4-h-old HSF2 embryos were probed with sequences complementary to the major actin transcripts (1.7, 1.95 and 2.2 kb)³⁸, providing size markers for the *ftz* and *hsp70-ftz* transcripts. Similar results were obtained using HSF3 embryos. Low levels of an additional 1.75-kb transcript were detected in all four lanes; whether this represents a *bona fide* *ftz* transcript is unknown.

in *ftz*⁻ embryos cannot be precisely demarcated, partly because of the lack of sufficient cuticular landmarks, but also because they are somewhat variable in extent (see Fig. 3 legend). Also, a small proportion of *ftz*⁻ embryos (usually <20%) show more extreme segmental fusions in which the denticle bands associated with one or more additional segments appear to be partially or completely deleted.

In contrast to *ftz*⁻ embryos, HSF2 and HSF3 embryos heat-shocked during the blastoderm stage can give rise to abnormal larvae which partially or completely lack the denticle bands normally formed by segments T1, T3, A2, A4, A6 and A8—exactly those denticle bands which are retained in *ftz*⁻ embryos (see, for example, Fig. 3). Thus, conditional, and presumably unrestricted, expression of the *hsp70-ftz* hybrid gene can cause a pair-rule phenotype which is superficially reciprocal to that resulting from absence of the *ftz* gene.

Both the frequency and extents of these reciprocal phenotypes are variable, perhaps because an extreme phenotype results only when the embryos are heat-shocked during a brief, optimal period during the blastoderm stage (for example, late enough for the *hsp70* promoter to be fully inducible, but early enough to allow sufficient *ftz* gene product to accumulate before it has to act). This variability has the fortunate consequence that a broad range of phenotypes can be examined. As in the case of

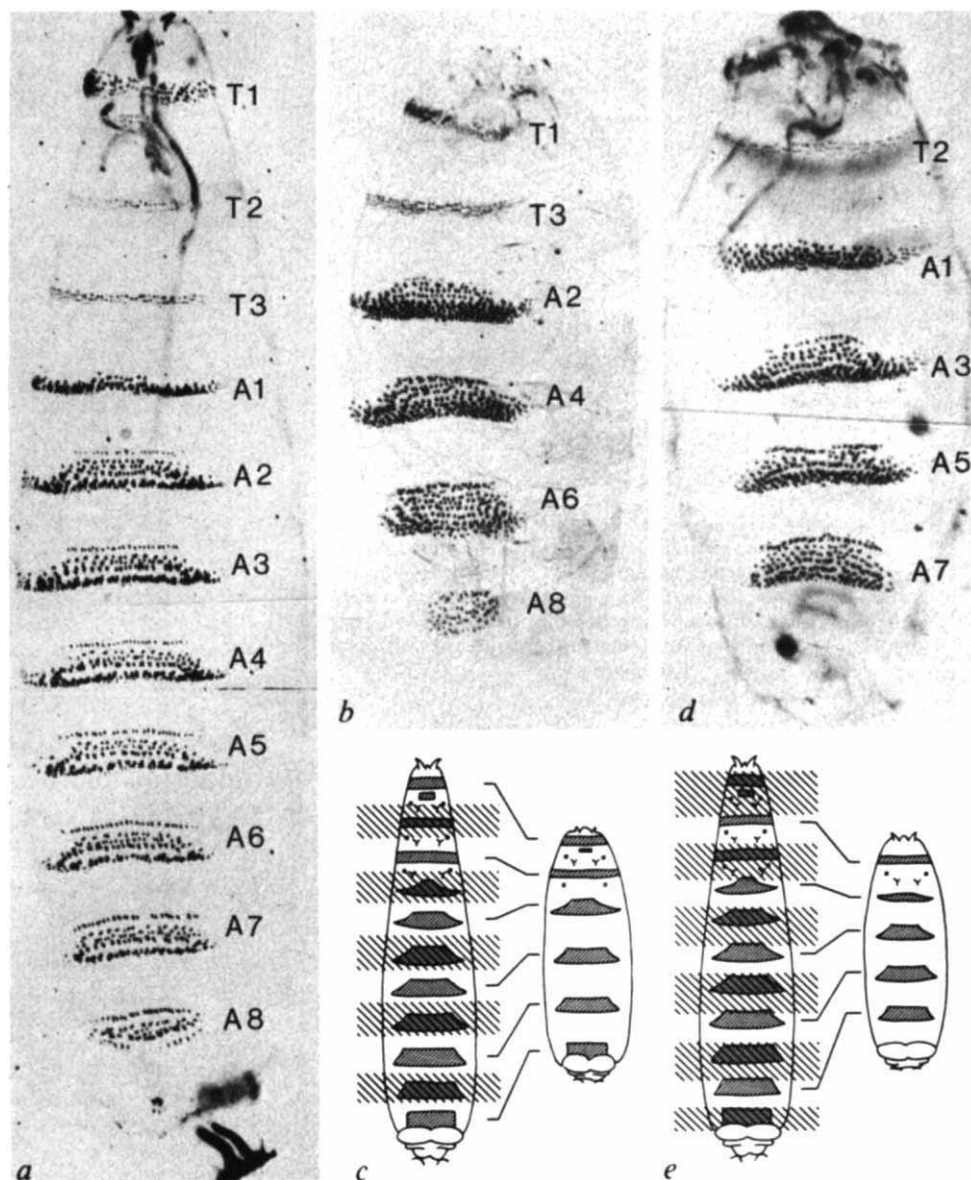
ftz⁻ embryos, there seems to be no absolute boundary between deleted and retained regions; however, the deletion pattern shown in Fig. 3e illustrates the boundaries which are usually respected. Note that the deletion patterns of *ftz*⁻ and heat-shocked HSF larvae are not perfectly reciprocal, but appear to overlap to some extent (Fig. 4).

The *ftz* gene is one of about 10 'pair-rule' genes that, when mutant, cause the apparent deletion or fusion of homologous portions of every other segment^{8–10}. Moreover, each of these genes is associated with a particular deletion pattern, suggesting that they are responsible for the development of overlapping, but different, repeating intervals of the segment pattern^{8–10}. The spatial distributions of transcripts of two pair-rule genes, *ftz* and *hairy* (*h*), have been determined; both are expressed in zebra patterns in which the active stripes correspond approximately to the regions of the body that are deleted in mutant embryos^{3,17}. The emerging picture therefore seems to be that the deletion patterns associated with the different pair-rule genes define alternating regions of the body where these genes are both expressed and required for normal development. Why then are the *ftz* and *h* genes (and possibly the remaining pair-rule genes) silent in the reciprocal portions of the body where their activities seem to be dispensable?

The results presented here argue strongly that the *ftz* gene is

Fig. 3 Near-reciprocal pair-rule phenotypes of *ftz*⁻ and heat-shocked HSF embryos. *a*, Wild-type first-instar larva. The three thoracic and first eight abdominal segments (T1–T3, A1–A8) each bear a characteristic band of ventral hairs (denticles) formed by the anterior compartment, *b*, *c*, *ftz*⁻ larva (homozygous for an apparent null allele, *ftz*⁹⁰⁹³; ref. 39). As described in the text, mutant larvae form only half the number of denticle bands (those belonging to segments T1, T3, A2, A4, A6 and A8)^{1,2} and hence appear to lack every other segment. In fact, the deleted regions are not segments, but rather segment-length units which begin close to the anteroposterior compartment boundary in one segment and end close to the boundary within the next segment. This is particularly clear in the thoracic segments because: (1) the patterns of dorsal and ventral hairs in each 'double segment' are composites of the anterior (a) and posterior (p) portions of adjacent segments (that is, T1a+T2p, T3a+A1p, A2a+A3p, etc.), (2) two sets of lateral sensory hairs⁴⁰ are often formed in each 'double segment' (normally each segment has a single set positioned just anterior to the compartment boundary within the segment), suggesting that a region slightly less than a segment's length lying between the lateral hairs in adjacent segments has been deleted, and (3) partial or complete Keilin's organs (which normally lie on or near the anteroposterior compartment boundary in each thoracic segment¹⁵) are usually present in the T1aT2p double segment, but are present only rarely in the T3aA1p double segment (deletion of a segment-length unit beginning and ending around the compartment boundaries within adjacent segments might be expected to leave behind partial or complete Keilin's organs more frequently when both segments are thoracic than when one is thoracic and the other abdominal). The exact boundaries between deleted and retained portions of the body seem to vary somewhat. For example, Keilin's organs are composed of three sensory hairs, two derived from the anterior compartment and a third derived from the posterior compartment¹⁵. In the T1aT2p double segment of *ftz*⁻ embryos, the Keilin's organs are sometimes rudimentary di-hairs or mono-hairs, or are absent. A further aspect of the *ftz*⁻ phenotypes is that some pattern elements, such as specific sensory hairs or rows of denticles, appear to be duplicated in each metameric unit². One consequence of this phenomenon, which has also been observed with mutations in other pair-rule genes⁴¹, is that the denticle bands appear broader than in wild-type embryos. *d*, *e*, Heat-shocked HSF3 larva. In contrast to *ftz*⁻ larvae, HSF larvae derived from embryos heat-shocked during the blastoderm stage often partially or completely lack the denticle bands associated with segments T1, T3, A2, A4, A6 and A8. Partial deletions often appear as fusions which join the denticle bands of particular pairs of segments (T1+T2, T3+A1, A2+A3, etc.). In progressively more extreme deletions, the denticle bands belonging to the more anterior segments (T1, T3, A2, etc.) appear progressively narrower or are eliminated, leaving the denticle bands derived from the more posterior segments (T2, A1, A3 etc.) largely intact (as in *d*). Thus, the deleted regions seem to be slightly larger than segment-length units which begin and end just outside the boundaries of segments T1, T3, A2, A4, A6 and A8. As in the case of *ftz*⁻ embryos, the limits of the deletion pattern illustrated in *e* seem to define the usual stopping line, but some embryos show more extreme deletion phenotypes. Because of the variation in phenotype, these limits should be considered provisional. *a*, *b*, *d*, Dark-field photomicrographs (ventral aspects, positive images; $\times 120$). *c*, *e*, The regions deleted from the normal pattern (after ref. 8). The denticle bands are shaded and the missing regions indicated by hatched bars; Keilin's organs and ventral pits are shown as Y and o symbols, respectively. Keilin's organs are found only rarely in the T3aA1p double segment of *ftz*⁻ larvae and are therefore not indicated in this position in *c*. No attempt has been made to map the deleted regions anterior to T1 or posterior to the A8 denticle band, though some portions of these terminal regions fail to develop in each case.

Methods. *d*, *e*, Embryos were collected and allowed to develop at 25 °C and were usually heat-shocked for 20 min at 35 °C, although shocks of 5 or 10 min were also effective at generating reciprocal *ftz* phenotypes. Typically, 500–1,000 fertilized eggs were collected during a 30-min period and shocked between 2½ and 3½ h after egg laying. In these conditions, ~50% of the resulting larvae showed partial or complete deletions of the denticle bands of at least three alternating segments; fewer than 1% of the embryos heat-shocked between 3½ and 3¾ h after egg laying (or at any time thereafter) developed into larvae showing reciprocal *ftz* phenotypes. HSF2 and HSF3 embryos responded similarly to heat shock.



normally silent in the 'off' stripes because it must be off to allow these regions to develop. Thus, the alternating on and off stripes of *ftz* activity seem to be equally critical, in their respective domains, for organizing the metamerous pattern of the body. Similar evidence is not yet available for the *h* gene. However, dosage studies of another pair-rule gene, *run*^{8,18}, have shown that extra wild-type copies can cause pair-rule deletions which are nearly reciprocal to the deletions resulting from loss of gene function (ref. 10 and J. P. Gergen and E. Wieschaus, personal communication). Though the interpretation is less clear in this

case, it is possible that these 'anti-run' phenotypes are caused by elevated levels of *run* expression in regions where the gene must normally be either silent or expressed at a low level.

These results can be explained by positing that the pair-rule genes act combinatorially to initiate the development of repeating portions of the segment pattern (see also ref. 10). For example, each cell along the anteroposterior axis of the blastoderm may express a specific combination of pair-rule genes (perhaps in response to a periodic spatial cue). This combination of genes then initiates the development of a specific interval of



Fig. 4 Overlap between the *fitz*⁻ and heat-shocked HSF pair-rule phenotypes. The regions of the body deleted in heat-shocked HSF larvae (Fig. 3e) are largely anterior to the regions deleted in *fitz*⁻ larvae (Fig. 3c). Note that the deletion patterns are not perfectly reciprocal: regions of common overlap (heavy shading) and common exclusion (unshaded) are found in alternating intervals along the body.

the repeat pattern, perhaps by activating the correct patterns of expression of other genes such as *engrailed*^{8,19-22} and the segment polarity genes^{8,9} which function subsequently to control growth and cell pattern within segments. Accordingly, both the on and off states of pair-rule gene function would have equal instructive roles because the developmental behaviour of any given cell or region would depend on the initial code-word of on and off pair-rule genes. In this regard, pair-rule genes may be acting in a manner functionally analogous to that of homoeotic genes of the bithorax and *Antennapedia* complexes²³⁻³¹.

Though this interpretation is prompted in part by the reciprocal nature of the *fitz*⁻ and heat-shocked HSF phenotypes, it should be noted, as shown in Fig. 4, that these phenotypes are not perfectly reciprocal: some regions of the body are deleted in both *fitz*⁻ and heat-shocked HSF larvae while other regions appear unaffected in either case. Regions of overlap between the two deletion patterns suggest that *fitz* and perhaps other pair-rule genes do not function simply as binary switches, but rather that differences in relative levels of their gene products, or perhaps temporal sequences of alternating expression, may also be important in defining specific portions of the segment pattern. Note that the stripes of cells expressing *fitz* transcripts narrow towards the end of the blastoderm stage^{3,32}, indicating that the gene may be on to different extents, or first on and then off, in some cells of each stripe.

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Simian virus 40-mediated *cis* induction of the *Xenopus* β -globin DNase I hypersensitive site

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Regions in chromatin which are hypersensitive to the action of DNase I appear to be associated with sites of genetic activity; the association between DNase I hypersensitivity and transcriptional activation is well known¹. In the case of the chicken β -globin gene the establishment of a DNase I hypersensitive site is dependent on tissue-specific *trans*-acting factors^{2,3}. Such factors have also been implicated in the action of viral and cellular enhancers⁴⁻¹⁰, which are themselves hypersensitive to DNase I¹¹⁻¹⁴. Enhancers have been defined operationally as DNA sequences which act in *cis* to potentiate transcription from their own, heterologous or cryptic promoters. This activity is essentially unaffected by changes in the orientation, position (5' or 3') or distance of the enhancer element with respect to its cognate promoter (ref. 15 for review). We demonstrate here that the transcriptional rescue of the *Xenopus laevis* β -globin gene by simian virus 40 (SV40) sequences including the enhancer coincides with the conferment of DNase I hypersensitivity upon that gene, and that this occurs in the absence of any change in the complement of *trans*-acting factors. These results suggest that a propensity to form sites hypersensitive to the action of DNase I is encoded in the primary sequence of DNA¹⁶, and that this predilection is aggravated by SV40 sequences, perhaps through a mechanism dependent on supercoiling.

Neither human nor rabbit β -globin genes are expressed when they are introduced by transfection into HeLa cells on plasmid vectors in a transient assay, but transcription of these genes is restored by linkage in *cis* to the SV40 enhancer^{17,18}. We have examined the behaviour of the *X. laevis* β_1 -globin gene in similar conditions. HeLa cells were transfected with recombinant plasmids and left for 48 h, after which both nuclei and total cytoplasmic RNA were prepared. The RNA was analysed for the presence of correctly initiated globin transcripts by quantitative S₁

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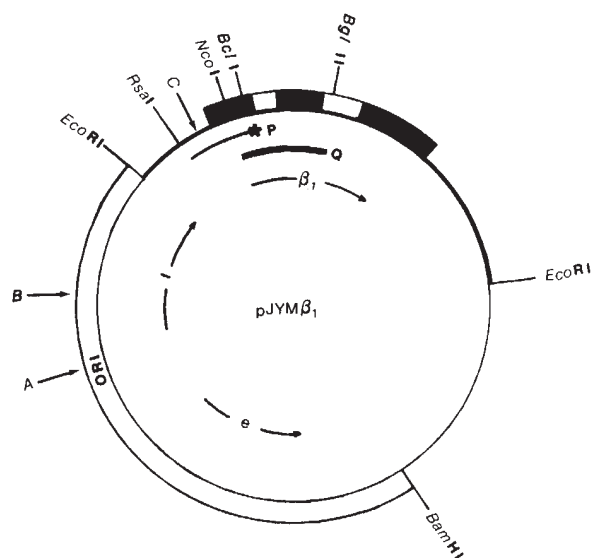


Fig. 1 The SV40-*Xenopus* β_1 -globin gene construct, pJYM β_1 . pXG β_1 (ref. 28) contains a 3.5-kilobase *EcoRI* *X. laevis* β_1 -globin gene fragment cloned into the *EcoRI* site of pAT153 (ref. 29). pJYM Δ RB was made by ligating the large *EcoRI*/*Bam*HI fragment containing all the early and part of the late transcription units of SV40 to an *EcoRI*/*Bam*HI fragment from pBR322 from which sequences poisoning replication in eukaryotic cells had been deleted³⁰. The *EcoRI* fragment containing the 3.5-kb β_1 -globin gene from pXG β_1 was then inserted into the unique *EcoRI* site of pJYM Δ RB to produce pJYM β_1 . The open box represents SV40-derived sequences. The thick line represents *X. laevis*-derived sequences. The thin line represents plasmid sequences. Arrows indicate the direction of e (early), l (late) and β_1 (globin) transcription. Approximate locations of DNase I hypersensitive sites A, B and C (Fig. 3) are arrowed. P is the 5' end-labelled *Bcl*I/*Rsa*I S_1 mapping probe. Q is the *Bgl*II/*Nco*I probe used for hypersensitive site mapping. ORI marks the approximate location of the SV40 origin.

nuclease mapping with the following results. When introduced alone (pXG β_1 ; Fig. 1 legend), the *X. laevis* β_1 -globin gene was not expressed (Fig. 2a, lane 6). Expression was not rescued by using DNA templates prepared from strains of *Escherichia coli* deficient in methylation (Fig. 2a, lane 7). However, when the gene was inserted into plasmid pJYM Δ RB (Fig. 1 legend), containing SV40 sequences, the resultant recombinant, pJYM β_1 (Fig. 1), produced high levels of β_1 -globin gene transcription (Fig. 2a, lane 5). The pattern of transcription obtained from pJYM β_1 was identical to that seen when RNA isolated from *X. laevis* erythroblasts was mapped using S_1 nuclease (Fig. 2a, lane 2). Furthermore, globin-specific RNA isolated from pJYM β_1 -transfected HeLa cells co-migrated on Northern blots with β_1 -globin messenger from *Xenopus* erythrocytes. Therefore, like the rabbit β -globin gene transcripts¹⁷, transcripts from the *X. laevis* β_1 -globin gene are correctly processed in HeLa cells.

Transcription could not be restored by co-transfecting pJYM Δ RB and pXG β_1 (Fig. 2b, lane 3), thereby demonstrating that it is not a result of the action in *trans* of SV40 early gene products. HeLa cells are semi-permissive for SV40¹⁹, and thus allow large-T-antigen-dependent replication of DNAs containing functional SV40 origins. Both pJYM Δ RB and pJYM β_1 produce large-T antigen as judged by immunofluorescence (data not shown), and have intact SV40 origins, thereby allowing them to replicate. pXG β_1 , on the other hand, is devoid of both the SV40 origin and early region. Although we have not formally excluded the possibility that transcriptional rescue is a result of replication, other workers¹⁷ have demonstrated that the enhancer-mediated potentiation of gene transcription is independent of this effect. We therefore consider it likely that the enhancer element in pJYM β_1 is involved in stimulating the expression of the *X. laevis* β_1 -globin gene.

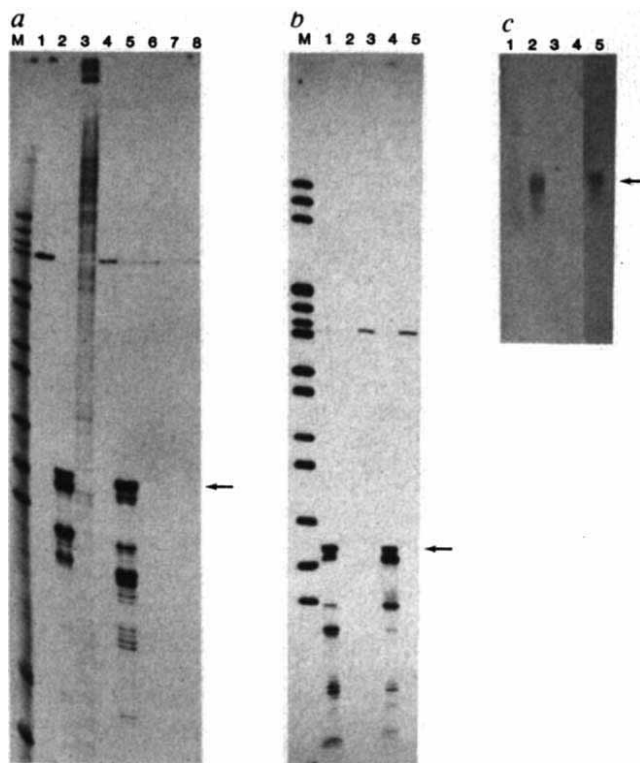


Fig. 2 Analysis of β_1 -globin gene expression in HeLa cells. Total cytoplasmic RNA isolated from HeLa cells 48 h after transfection was analysed for *X. laevis* globin-specific transcription by either S_1 nuclease mapping^{31,32} followed by electrophoresis on denaturing polyacrylamide gels³³ (a, b), or Northern blotting³⁴ (c). The size marker, M in a and b, is a 3'-end-labelled pAT153 *Hpa*II digest yielding fragment sizes of : 634 (faint), 494 (faint), 407 (faint), 244, 240, 219, 205, 192, 162 (doublet), 149, 124, 112, 92, 78, 69, 36 and 28 base pairs (bp). a, Lane 1, 190-bp *Bcl*I/*Rsa*I fragment used as S_1 probe (P in Fig. 1). Lane 2, 225 ng of total cytoplasmic RNA from X.1. erythroblasts. The arrow indicates the protected fragment size for authentic globin mRNA. Lane 3, a Maxam and Gilbert sequencing reaction size marker. Lane 4, pJYM Δ RB. Lane 5, pJYM β_1 . Lane 6, pXG β_1 . Lane 7, pXG β_1 grown in a *dam*⁻ strain of *E. coli*. Lane 8, 5 μ g of transfer RNA. b, Lane 1, pJYM β_1 . Lane 2, pXG β_1 . Lane 3, pJYM Δ RB + pXG β_1 . Lane 4, 225 ng of total cytoplasmic RNA from X.1. erythroblasts. Lane 5, 5 μ g of transfer RNA. The arrow indicates the protected fragment size for authentic β -globin mRNA. c, Lane 1, pJYM Δ RB. Lane 2, pJYM β_1 . Lane 3, pXG β_1 . Lane 4, pXG β_1 *dam*⁻. Lane 5, 225 ng of total cytoplasmic RNA from X.1. erythroblasts. The arrow indicates the size of processed *X. laevis* β_1 -globin mRNA. **Methods.** Transfection and RNA preparation: HeLa cells were transfected by the DEAE-dextran procedure essentially as described elsewhere³⁵ with the following modifications: (1) after removal of the DNA/DEAE-dextran mixture, cells were 'shocked' with 5 ml of phosphate-buffered saline containing 17% glycerol for 1 min; and (2) cells were not treated with chloroquine diphosphate after transfection. After 48 h, the cells were lysed as described in Fig. 3 legend, and total cytoplasmic RNA was prepared as described elsewhere³⁶ and run on neutral agarose gels. Ribosomal RNA profiles were used to evaluate RNA concentration and integrity. S_1 nuclease analysis: The *Bcl*I/*Rsa*I fragment (P in Fig. 1) of the *X. laevis* β_1 -globin gene was 5'-end-labelled with ³²P to a specific activity of 1×10^7 c.p.m. per pmol of 5' ends, strand separated on denaturing polyacrylamide gels and electroeluted³³. Cytoplasmic RNA (15 μ g) isolated from transfected HeLa cells was hybridized to 2×10^5 c.p.m. of probe (probe excess) as described elsewhere³⁷, and digested with S_1 nuclease as described previously³² except that the concentration of Zn^{2+} was increased by a factor of 10 to compensate for the high level of EDTA in the hybridization buffer. Northern analysis: Cytoplasmic RNA (15 μ g) isolated from transfected HeLa cells was electrophoresed on a formaldehyde/agarose gel, transferred to nitrocellulose³⁸ and hybridized with a nick-translated³⁹ *Xenopus* β_1 -globin cDNA clone pXG8D2⁴⁰ as described previously³⁴.

As DNA transfected into cultured cells has been shown to form chromatin²⁰, we compared the chromatin structure of the transcriptionally active globin gene in pJYM β_1 with that of the quiescent copy in pXG β_1 . Given the close correlation between DNase I hypersensitivity and transcriptional activity¹, we chose DNase I as a structural probe. Nuclei from transfected cells were treated with differing concentrations of DNase I, after which total DNA was prepared and analysed by indirect end labelling²¹. This analysis revealed three hypersensitive sites in pJYM β_1 , designated A, B and C (Fig. 3). Site A maps to the late side of the SV40 control region and probably corresponds to the reported DNase I hypersensitivity of the enhancer element itself^{14,20}. Site B occurs in the SV40 late coding region between the initiation codons for viral capsid proteins VP2 and VP3. This site has not been described previously, although the data of Jongstra *et al.*²² reveal a hypersensitive band in the same region. Site C occurs in the 5' region of the *X. laevis* β_1 -globin gene extending roughly from -100 to +50 relative to the start of transcription at +1. This agrees quite closely with the position of the hypersensitive site associated with the *X. laevis* β_1 -globin gene in *Xenopus* erythrocytes (ref. 23 and J. Allan, unpublished). None of these sites is observed when naked DNAs are subjected to DNase I (M. E. Walmsley *et al.*, in preparation).

Note that when no DNase I was added, pJYM β_1 was specifically cleaved by an endogenous nuclease (Fig. 3, lane 6). It can be seen that the three DNase I hypersensitive sites form only a small subset of the regions recognized by the endogenous nuclease. The endogenous cleavage pattern may reflect some other feature of chromatin structure; alternatively, these bands may be the products of cleavage of naked DNA molecules with a marked sequence specificity, a possibility now being investigated in our laboratory. DNase I analysis of nuclei from HeLa cells transfected with pXG β_1 revealed no hypersensitive sites (Fig. 3, lanes 1-5); this was not due to a failure of DNase I, whose activity was confirmed by the reduction in size of HeLa cell genomic DNA as judged by ethidium bromide staining (data not shown).

The concomitant rescue of *Xenopus* β_1 -globin transcription and the induction of hypersensitivity seen in pJYM β_1 was also achieved in another construct, pJYM $\beta_1\Delta 1p$ (Fig. 4). In this construct the late promoter region of SV40 has been deleted, excluding the possibility that the results obtained with pJYM β_1 were due to activation of the globin unit by 'readthrough' late transcription. Furthermore, the orientation of the *Xenopus* β_1 -globin gene relative to the viral early control region has been changed in pJYM $\beta_1\Delta 1p$, reinforcing the long-range nature of the process(es) involved. This could be taken as evidence for a causative role for the SV40 enhancer, which is known to act essentially independently of orientation and distance. However, like pJYM β_1 , pJYM $\beta_1\Delta 1p$ can replicate in HeLa cells. We have not excluded a possible role for replication, whose effects are also relatively position independent, in the activation of the *Xenopus* β_1 gene.

The results presented here reinforce the already strong correlation between DNase I hypersensitivity and transcriptional activity¹. Their novelty resides in the fact that a DNase I hypersensitive site is established by changing the sequence environment of a gene without any concomitant alteration in the complement of cellular *trans*-acting factors. It is unlikely that DNase I hypersensitivity is merely a consequence of transcription, for in a chicken erythroid cell line, DNase I hypersensitivity of the β -globin gene precedes its transcriptional activation²⁴. Our results are particularly pertinent given the association between the binding of *trans*-acting factors and hypersensitivity¹⁵, together with the demonstration that the reconstitution *in vitro* of the chicken globin gene DNase I hypersensitive site is dependent on development- and tissue-specific factors^{2,3}. Weintraub, however, has proposed¹⁶ that the nuclease-hypersensitive 5' and 3' regulatory regions of eukaryotic genes have the potential to form an altered DNA secondary structure, and that this ability is contained within the DNA sequence alone. He demonstrated

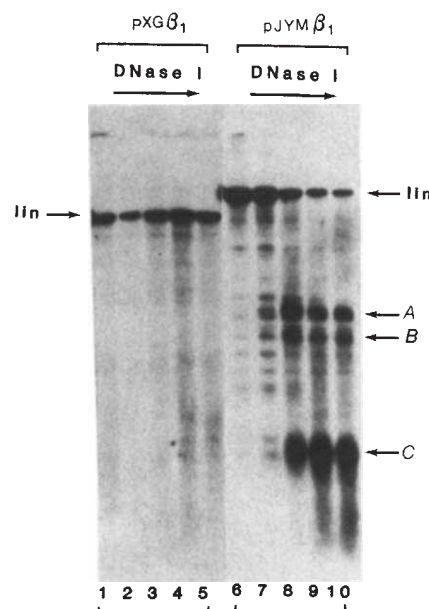


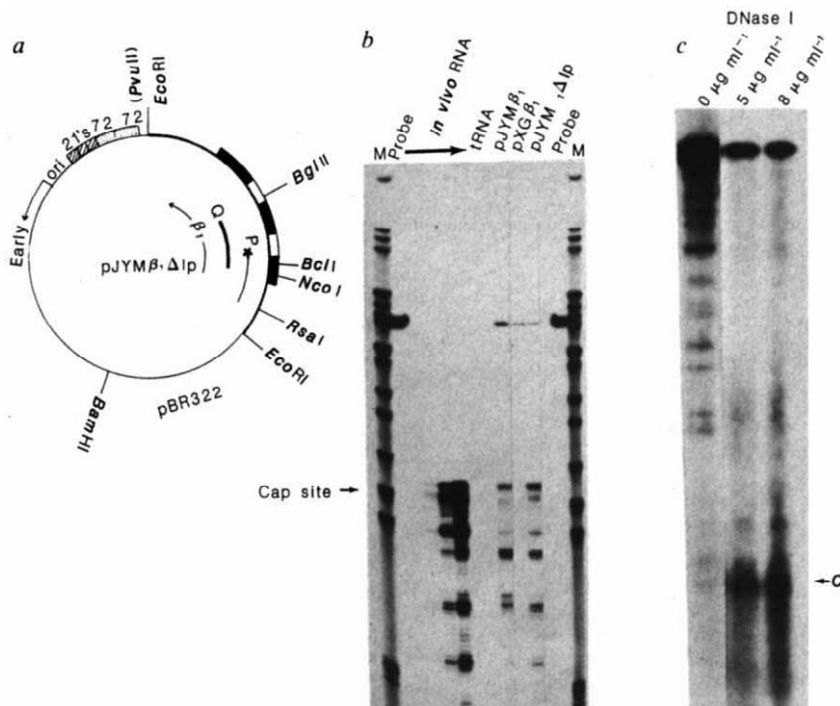
Fig. 3 Indirect end-labelling analysis of DNase I-treated transfected material. Nuclei were prepared from transfected HeLa cells after 48 h and treated with DNase I. Total DNA was then restricted with *Bgl*II and hybridized with a *Bgl*II/*Nco*I probe (Q in Fig. 1). The linear main band is marked 'lin' and hypersensitive sub-bands are arrowed A, B and C. Lanes 1-5 represent nuclei from cells transfected with pXG β_1 treated with 0, 1, 2, 5 and 8 $\mu\text{g ml}^{-1}$ DNase I (Worthington) respectively. Lanes 6-10 represent the same concentration course performed on nuclei from cells transfected with pJYM β_1 .

Methods. Trypsinized HeLa cells from a confluent 250-ml flask were washed once in RPMI 1640 medium containing 20 mM HEPES, resuspended in 0.72 ml of ice-cold lysis mix (50% glycerol, 50 mM Tris 7.9, 100 mM KCl, 5 mM MgCl₂, 0.05% saponin and 200 mM β -mercaptoethanol) and left on ice for 10 min. The lysed mixture was spun at 5,500 r.p.m. for 15 min at -5°C in a Sorvall HB4 fitted with Eppendorf tube adaptors. The supernatant was removed for RNA preparation and the nuclear pellet was resuspended in 0.8 ml of ice-cold buffer A (100 mM NaCl, 50 mM Tris pH 8.0, 3 mM MgCl₂, 0.1 mM phenylmethylsulphonyl fluoride), then repelleted in the same rotor assembly for 5 min at 3,000 r.p.m., 0°C . The nuclear pellet was then resuspended at 500 $\mu\text{g ml}^{-1}$ in ice-cold buffer A. To 18- μl aliquots of this was added 2 μl of differing dilutions of DNase I in buffer A; these reactions were incubated for 30 min at 37°C before termination by addition of 1 μl of 0.5 M EDTA pH 8.0. RNase was added to a final concentration of 20 $\mu\text{g ml}^{-1}$ and after a further 30-min incubation at 37°C , proteinase K and SDS were added to 50 $\mu\text{g ml}^{-1}$ and 0.25%, respectively, and the samples left overnight at 37°C . Sample volumes were increased to 100 μl before extraction with phenol/chloroform and chloroform, followed by ethanol precipitation. Resuspended DNAs were restricted overnight with *Bgl*II, electrophoresed on 0.8% agarose gels, transferred to nitrocellulose⁴¹ and hybridized with a ^{32}P -labelled *Bgl*II/*Nco*I fragment (Q in Fig. 1) prepared as described elsewhere⁴².

that these secondary structures observed *in vivo* may be elicited *in vitro* by DNA supercoiling.

We speculate that our observations are best interpreted by a mechanism involving both *cis*- and *trans*-acting components, and envisage a scenario whereby the SV40 early control region binds a *trans*-acting factor(s) which in turn direct(s) the binding of a DNA gyrase-like activity that induces DNA supercoiling. DNA sequences in the proximity of the globin hypersensitive site, with a propensity to form altered secondary structures under superhelical stress, adopt conformations which directly or indirectly discourage the placement of nucleosomes at the hypersensitive site. Supercoiling has already been implicated in generalized DNase I sensitivity²⁵ as well as transcriptional activity²⁶. This, together with the recent observation that the SV40 enhancer region is the major site of action of drug-induced cleavage by

Fig. 4 Analysis of a SV40-*Xenopus* recombinant genome lacking the viral late region. *a*, Map of pJYM $\beta_1\Delta 1p$. This construct is identical to pJYM β_1 except that the *EcoRI*/*PvuII* fragment containing viral late sequences has been removed, and the *Xenopus* globin gene fragment has been inverted. *b*, *S*₁ nuclease analysis of RNA isolated from HeLa cells transfected with pJYM $\beta_1\Delta 1p$. Probes, markers and methods as for Fig. 2. The *in vivo* RNA lanes contain 1.0, 10, 100 and 1,000 ng of total cytoplasmic RNA isolated from *X. laevis* erythroblasts. *c*, DNase I analysis of nuclei isolated from HeLa cells transfected with pJYM $\beta_1\Delta 1p$. Methods as for Fig. 3. The hypersensitive site of the *Xenopus* β_1 -globin gene is marked C.



mammalian DNA topoisomerase II²⁷, lends credence to this model. Whatever the mechanism of the induction, our data clearly demonstrate that hypersensitive sites can be induced in *cis*.

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Assembly of microtubules from nucleotide-depleted tubulin

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In vitro assembly of microtubules from tubulin is considered to have an absolute requirement for added GTP (or a non-hydrolysable GTP-analogue)¹ involving binding at the E(exchangeable)-site located on the β -subunit of the tubulin dimer². By contrast, GDP inhibits assembly. Nucleotide hydrolysis has been implicated in the dynamic properties of microtubules^{3,4}, treadmilling⁵ and mechanical coupling⁶. Here we demonstrate that assembly is not necessarily dependent on the presence of GTP at the E-site; microtubules can be formed efficiently in the absence of GTP in the presence of pyrophosphate. These microtubules, which have normal morphology and lability at cold temperatures, contain N(non-exchangeable)-site GTP and a significant proportion of E-site GDP. This demonstrates the possibility of direct incorporation of GDP-containing tubulin dimer during assembly which probably derives from microtubule-associated protein (MAP)-containing oligomers. This finding has important implications for the mechanism of microtubule elongation. The effects of pyrophosphate suggest that charge neutralization by the bidentate ligand is an essential step in promoting microtubule assembly, and that this interaction involves only a minimal conformational change in the protein.

Studies of nucleotide-depleted tubulin are restricted by high nucleotide affinities⁷ and protein instability⁸⁻¹⁰. We have quantitated the removal of E-site GDP by alkaline phosphatase¹¹, using HPLC to follow nucleotide hydrolysis. The progressive loss of GDP (at 20 °C) is faster and more extensive from tubulin dimer than from microtubule protein (tubulin+MAPs). After treatment for 25 min, the assembly capability of this microtubule

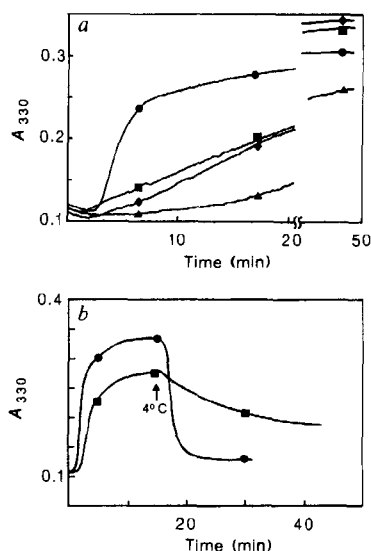


Fig. 1 Pyrophosphate-induced assembly of nucleotide-depleted microtubule protein at 37 °C in the absence of GTP. *a*, Absorbance (330 nm) of microtubule protein (1.2 mg ml⁻¹) treated with alkaline phosphatase for various times at 20 °C, before addition of 3 mM pyrophosphate and incubation at 37 °C in the absence of GTP. The protein was incubated with alkaline phosphatase for 0 (▲), 10 (■) and 25 (●) min. Control (○), no fast assembly is observed after treatment with alkaline phosphatase for 25 min in the absence of pyrophosphate. *b*, Assembly (37 °C) and disassembly (4 °C) of microtubule protein in 1 mM GTP (●) and of alkaline phosphatase-treated microtubule protein in 3 mM pyrophosphate (■). Protein concentration, 1.2 mg ml⁻¹.

protein (on re-addition of 1 mM GTP, plus 3 mM pyrophosphate to inhibit further alkaline phosphatase action) is >90%, showing that nucleotide removal is reversible.

If pyrophosphate is added after alkaline phosphatase treatment (but without subsequent addition of GTP) a specific pyrophosphate-induced microtubule assembly is observed (Fig. 1). Pyrophosphate added to the nucleotide-depleted microtubule protein (containing 40% E-site GDP) causes a rapid increase in turbidity at 37 °C (Fig. 1*a*, circles), superimposed on the more gradual background increase. The rapid process is caused by microtubule formation (see below); it does not occur if the pyrophosphate is either omitted or added for shorter periods (Fig. 1*a*). Neither phosphate, sulphate nor triphosphate can substitute for pyrophosphate. There is maximal effect at 3–5 mM pyrophosphate, whereas higher concentrations of pyrophosphate are inhibitory. Although prior addition of 1 mM GDP inhibits the rapid effect, there is little effect with addition at plateau: the pyrophosphate-induced assembly does show partial cold lability (Fig. 1*b*).

To eliminate potential effects of dephosphorylation of MAPs by alkaline phosphatase, nucleotide-depleted tubulin dimer was prepared from phosphocellulose-purified tubulin by treatment with alkaline phosphatase for 15 min; the resultant tubulin contained ~20% E-site GDP. Following addition of heat-treated intact MAPs¹² (not exposed to alkaline phosphatase), the nucleotide-depleted tubulin dimer assembled at 37 °C with 3 mM pyrophosphate.

Electron microscopy shows microtubules of normal morphology in the pyrophosphate-induced assembly. The microtubules show well-defined protofilament structure and the rough-walled appearance characteristic of the presence of MAPs (Fig. 2*a*). These microtubules are distributed uniformly throughout the samples (Fig. 2*b*). After disassembly at cold temperatures only amorphous material is seen. When alkaline phosphatase-treated microtubule protein is incubated at 37 °C without pyrophosphate, similar amorphous material is produced (Fig. 2*c*). The microtubules are relatively short compared with those assembled with microtubule protein plus GTP. Micro-

tubules of normal morphology are also formed from nucleotide-depleted tubulin dimer with MAPs as described above (Fig. 2*d*).

The nucleotide content of the pyrophosphate-induced microtubules was analysed by HPLC (Table 1). The initial Sephadex G-25 treated protein contains 1/1 molar ratio GTP/GDP; the E-site nucleotide is >97% GDP. The GTP content indicates tightly bound nucleotide at the N-site, which serves as a marker of the proportion of tubulin retaining structural integrity. Alkaline phosphatase removes GDP from the E-site. On resuspension of the 37 °C microtubule pellet at 0 °C, 86% of the nucleotide-containing tubulin appears in solution, and thus derives from cold-disassembled nucleotide-depleted microtubules. From the GDP/GTP ratio, these microtubules contain nucleotide in the proportion 0.5 mol E-site GDP per mol N-site GTP. Using ³H-GDP, this GDP is shown to derive entirely from E-site GDP bound to tubulin before assembly.

Our results show that nucleotide-depleted microtubule protein can be assembled in the absence of GTP into morphologically

Table 1 Nucleotide content of pyrophosphate-induced microtubules assembled at 37 °C from nucleotide-depleted microtubule protein in the absence of added GTP

	GTP	GDP	GMP/Guo	Total
Microtubule protein (Sephadex G-25 eluate):	100	94	4	(198)
After alkaline phosphatase treatment and assembly at 37 °C:				
S1	86	38	75	(199)
P1	52	20	78	
P2	29	14	3	(196)
0 °C resuspension of P1:				
S2	25	12	0	
P2	0	0	0	
Ratio S2/P1 (%)	86	86		

Alkaline phosphatase (40 U ml⁻¹) was added to 2.0 mg ml⁻¹ Sephadex G-25-treated bovine microtubule protein¹⁵ (25 min at 20 °C), followed by addition of 3 mM pyrophosphate. After incubation at 37 °C for 15 min, centrifugation (for 5 min at 140,000g in the Airfuge; A100 rotor) of the sample gave a supernatant (S1) and pellet (P1). After resuspension of P1 at 0 °C for 30 min in MEM100 buffer¹⁵, centrifugation gave supernatant S2 and pellet P2. The nucleotide content (mean data of seven experiments), was assessed by HPLC and is expressed relative to initial N-site GTP as 100%; bracketed figures indicate total nucleotide recovery at different stages. Electrophoresis and densitometry of Coomassie blue-stained SDS-polyacrylamide 7.5% gels shows that 47% total protein is pelleted in P1; this material shows a normal ratio of MAPs to tubulin (16% HMW1 and 2). Guo = guanosine.

normal, cold-labile microtubules. There is no evidence that contaminating GTP is responsible for the assembly. We observe short microtubules, indicating efficient nucleation; pyrophosphate is implicated in both the nucleation and elongation processes.

It is striking that the pyrophosphate-induced assembly of nucleotide-depleted microtubule protein gives microtubules which contain substantial proportions of tubulin-GDP. The mechanism of tubulin-GDP incorporation during elongation of microtubules is controversial^{1,13}. Isotope labelling¹⁴, assembly kinetics¹⁵ and time-resolved X-ray scattering¹⁶ techniques indicate direct incorporation of oligomeric fragments into assembling microtubules. The E-site GTP/GDP content of assembling oligomeric fragments is not well-defined. We have shown, however, that in nucleotide-depleted microtubule protein the GDP-tubulin is entirely associated with MAP-containing oligomers. Our present work therefore raises the possibility that fragments containing substantial proportions of tubulin-GDP could be incorporated as such, that is, without prior conversion to tubulin-GTP. We have recently demonstrated¹⁷ that this occurs for the assembly of microtubule protein induced by the GTP analogue GMPPCP. This indicates a minimum requirement for GTP (or an analogue such as GMPPCP or pyrophosphate), possibly at the elongation site, but not necessarily within the

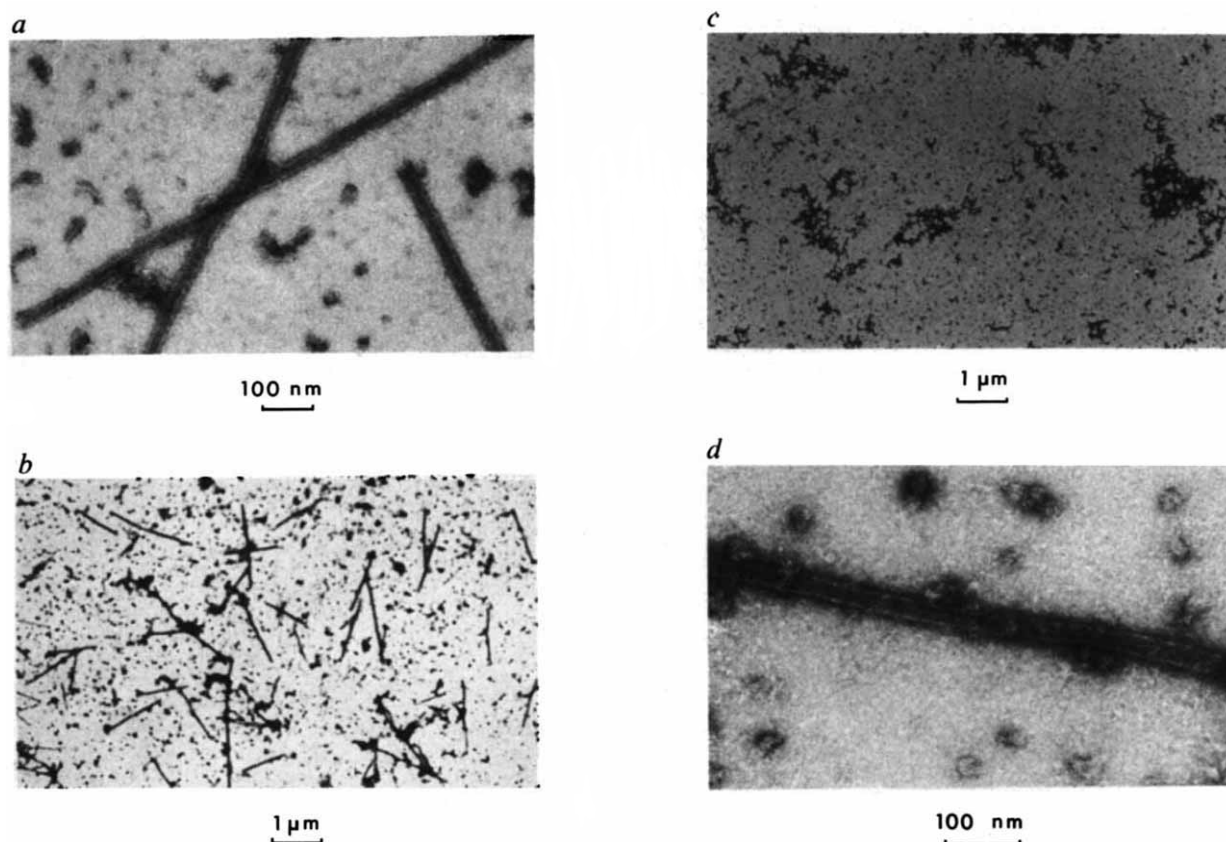


Fig. 2 Electron micrographs of products of assembly with nucleotide-depleted microtubule protein or tubulin plus MAPs at 37 °C with pyrophosphate. Nucleotide-depleted protein was prepared by treatment with alkaline phosphatase at 20 °C as described in Fig. 1 legend; microtubule protein (*a-c*) was incubated for 25 min, tubulin dimer (*d*) for 15 min. *a*, Addition of 3 mM pyrophosphate before 37 °C incubation; *b*, low-magnification view of *a*; *c*, control 37 °C, no pyrophosphate; *d*, nucleotide-depleted tubulin dimer plus normal MAPs (see text) incubated at 37 °C with 3 mM pyrophosphate. (Samples *a* to *d* were prepared for electron microscopy by negative staining as described in ref. 15.)

oligomeric fragment itself. Thus, the nucleotide requirements for assembly of microtubule protein appear to be less stringent than those for pure tubulin dimer.

Direct interaction of pyrophosphate with tubulin has been shown by competition experiments with ^3H -GDP (unpublished data). As a mechanism for pyrophosphate-induced assembly, we propose that this ion binds to tubulin at the E-site in locations corresponding to the high-affinity sites for β - γ positions of GTP. The pyrophosphate ion promotes assembly of nucleotide-depleted tubulin without inducing major secondary or tertiary structural changes^{18,19}. The adoption of an assembly-competent state of tubulin could therefore require no more than the simultaneous occupancy of two potentially adjacent positively charged sites by an appropriate bidentate negatively charged ligand, of which pyrophosphate is a simple example. We suggest that by such an alignment of two protein domains, a minimal protein conformational change facilitates the required interactions for polymerization.

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The CO bond angle of carboxymyoglobin determined by angular-resolved XANES spectroscopy

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Our knowledge of the structure of condensed matter has been based primarily on spectroscopic methods that measure first-order pair correlations of atomic arrangements and thus provide interatomic distances (for example neutron and X-ray scattering). Bond angles are given by higher-order correlation functions, and such information can be provided by X-ray absorption near-edge structure (XANES) spectroscopy^{1,2}, the features of which are determined by multiple scattering of photoelectrons whose paths

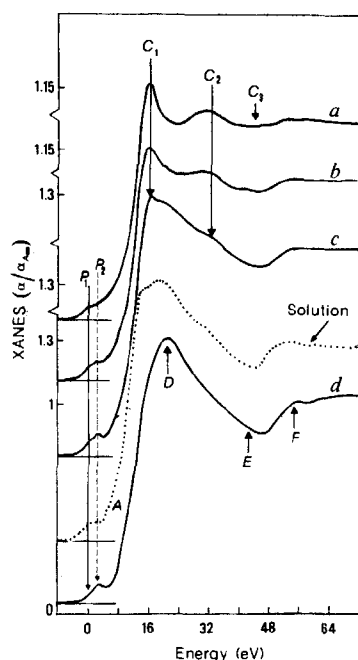


Fig. 1 Experimental XANES spectra (a-d) of a single MbCO crystal for different orientations of the crystal with respect to the polarization vector $\vec{E}$. The crystal was rotated from the $\vec{E} // \hat{a}^*$ (curve a) to the $\vec{E} // \hat{c}$ (curve d) orientation. The calculated angles φ between $\vec{E}$ and the haem normal $\hat{n}$ are: a, 28° ; b, 41° ; c, 62° ; d, 85° . The experimental XANES spectrum of MbCO in solution is shown dotted.

begin and end at the selected absorbing atom. We report here angular-resolved XANES spectroscopy of a single crystal of carboxymyoglobin (MbCO). The large dichroism of the X-ray absorption of the crystal can be fully interpreted by multiple-scattering theory which allows the determination of Fe-ligand bond angles. The analysis of the identified multiple scattering features due to CO in high signal-to-noise-ratio spectra of protein in solution has allowed the determination of the variation of CO bond angles. This opens the way to the determination of subtle structural features due to bond angle variations in proteins in solution which are relevant to an understanding of the characteristics of proteins at the atomic scale.

Knowledge of Fe-C-O and Fe-O₂ bond angles in solution is fundamental to the understanding of reversible molecular binding of diatomic molecules in haem proteins, but current data are limited to results obtained by crystallography. In fact, only infrared CO stretching and ¹³C nuclear magnetic resonance (NMR) spectroscopy are available to probe CO bonding in proteins in solution; moreover, many factors affect the spectroscopic signals and no simple interpretation has been established in terms of protein structure^{3,4}. XANES spectroscopy is useful in that it is a direct structural probe which can be used to study both crystals and solutions.

We first applied XANES spectroscopy to a MbCO single crystal, a system which has been extensively studied by crystallography. The photoelectron multiple scattering resonances in the direction of CO have been identified which probe the three-atom correlation function and therefore give the C-O bond angle. The Fe-C-O configuration has been found to be bent, with a Fe-C-O bond angle of $\sim 150^\circ$; this agrees qualitatively with neutron diffraction data⁵, although that technique is subject to large errors.

We then used the XANES method to study the Fe-C-O bond angle in haem proteins in solution. The infrared CO stretching signal in MbCO changes on going from crystal to solution⁶, and this has led to the suggestion that the FeCO configuration is different in solution. On the other hand, La Mar *et al.*⁷ found no difference between the ¹³C-NMR signal of MbCO and the

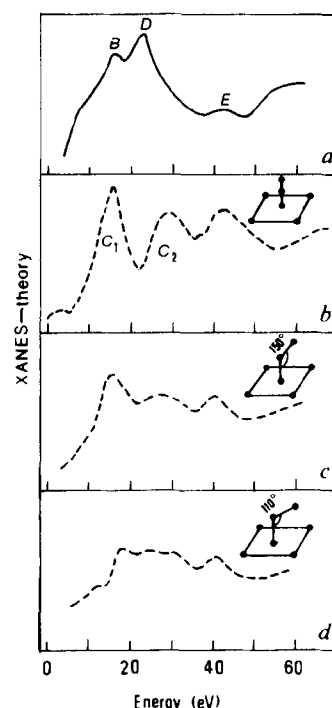


Fig. 2 Theoretical angular resolved XANES. a, The experimental features A, B, D, E and F due to the scattering in the haem plane, $\vec{E} \perp \hat{n}$, are predicted by the theory. b-d, The $\vec{E} // \hat{n}$ XANES curves for various CO bond angles. The best agreement between the theoretical and experimental spectra was found for the Fe-C-O configuration at $\theta = 150^\circ$.

unhindered synthetic haem model, and suggested that there was no distortion of the FeCO configuration in MbCO in solution. This is in contrast to the bent configuration observed in crystals. We have found that MbCO in solution shows a Fe-C-O configuration that is bent at $\theta \approx 150^\circ$, as occurs in the crystal. However, for carboxyhaemoglobin (HbCO) in solution we found $\theta = 165^\circ$ whereas crystallographic studies of HbCO crystals found a smaller value of $\theta = 136^\circ$ (ref. 8).

A large ($5 \times 3 \times 0.8$ mm) MbCO single crystal (space group P2₁) was oriented in a collimated X-ray synchrotron radiation beam completely intercepted by the crystal. The XANES spectra were measured by recording the intensity of the FeK α fluorescence as a function of the X-ray photon energy, and the crystal was rotated in steps around the b-axis from the $\vec{E} // \hat{a}^*$ to the $\vec{E} // \hat{c}$ orientation, where $\hat{a}$ and $\hat{c}$ are the crystallographic axes and $\vec{E}$ is the polarization vector. The data on the crystals were recorded using synchrotron radiation sources at Daresbury Laboratory and those on the 5 mM HbCO, MbCO and MbCN solutions were recorded at Frascati Laboratory.

Figure 1 shows the angular resolved FeK XANES of the MbCO single crystal. The polarized X-ray absorption spectra can be classified according to the angle φ between $\vec{E}$ and the haem normal axis $\hat{n}$. The MbCO spectrum for $\vec{E} // \hat{a}^*$ ($\varphi = 28^\circ$) is mostly due to multiple scattering along the haem normal and for $\vec{E} // \hat{c}$ ($\varphi \sim 86^\circ$) the spectrum is mostly due to photoelectron multiple scattering in the haem plane. Features C₁ and C₂ clearly arise from photoelectron multiple scattering along the normal direction, while features A, B, D, E and F arise from scattering in the haem plane. All are present in the spectrum of MbCO in solution (Fig. 1).

The theoretical FeK XANES of MbCO was calculated using the full multiple scattering approach (refs 2, 9-11 and S. Hasnain, A.B. and S.P., unpublished), in which the absorption cross-section for one-electron excitations from a core level is determined by the sum of all scattering paths for the photoelectron which begin and end on the same absorbing Fe atom at the origin. Figure 2 shows several theoretical angular-resolved XANES spectra of MbCO. The atomic coordinates of the cluster

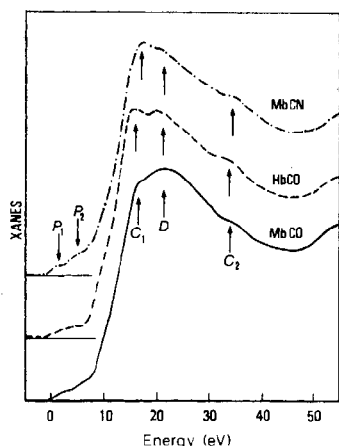


Fig. 3 Experimental XANES spectra of MbCO, HbCO and MbCN in solution. The main difference is the variation of the intensity ratio between peak C_1 and peak D which depends on the bonding angle Fe-C-O(N).

of 30 atoms have been deduced from diffraction data⁵ and polarized extended X-ray absorption fine structure (Fe- $N_p = 2.0$ Å, Fe- $N_e = 2.09$ Å, Fe-C = 1.84 Å) (S. Hasnain, A.B. and S.P., unpublished), and an idealized symmetrical and plane porphyrin ring was used. Figure 2a shows that the theory predicts all the experimental features A, B, D, E, F due to the scattering in the haem plane, while Fig. 2b-d shows the large variation of the $E//\hat{n}$ spectra obtained by changing the CO bonding geometry. The best agreement between the theoretical spectrum and the experimental results was found for the Fe-C-O bent configuration at $\theta = 150^\circ$. Using the crystallographic coordinates, which have large errors⁵, with Fe-C at 1.78 Å and C-O parallel but displaced from the haem normal axis, we obtain a large disagreement with the XANES experiment.

Finally, having demonstrated the XANES method for a single crystal, we next applied it to determine the bonding geometry of ligands in haem proteins in solution, where diffraction methods cannot be applied. Figure 3 shows the Fe XANES spectra of MbCO, HbCO and MbCN in solution with good signal-to-noise ratio. The main difference between these spectra is the variation of the intensity ratio between peaks C and D , which depends strongly on the Fe-C-O(N) bonding angle. Both by calculation and by comparison with crystal XANES spectra, we find the Fe-C-O angle in solution to be $\sim 150^\circ$, as it is in MbCO crystals.

Theoretical XANES analyses indicate that the Fe-C-O(N) angle is 150° in MbCO, 165° in HbCO and 180° in MbCN in solution. Our results strongly support the Moffat hypothesis¹² that the CN configuration in HbCN is linear and tilted rather than bent. We find, within the sensitivity of the XANES method, that the Fe-C-O configuration is bent in both crystals and solutions, and we estimate that we can easily detect variations of $\Delta\theta = 10^\circ$. We have obtained experimental evidence that the (Fe-C=O) configuration is different in HbCO and MbCO in solution ($\Delta\theta = +15^\circ$ going from MbCO to HbCO) and therefore that the CO bonding is modified by the protein. Crystal diffraction studies have shown a different bent configuration, with $\theta = 136^\circ$ in HbCO (ref. 8). Our data therefore indicate that there is a difference in CO bonding in HbCO in the crystalline and solution phases.

In conclusion, our results show that there is a large dichroism in the X-ray range of the haem protein crystal which can be fully analysed and its bond angles determined quantitatively by XANES. This method can be used to improve our knowledge of the subtle variations that occur in the local structure of proteins and can thus help us to establish the relationship between localized structural changes and the mechanism of protein control of ligand binding in solution close to the protein native state.

We thank M. Perutz for discussions and hospitality at the MRC Laboratory in Cambridge where the MbCO crystal was prepared, and the synchrotron radiation facilities of the SERC Daresbury Laboratory and of the INFN-CNR Frascati Laboratory. This project has been partially supported by the office for international collaboration and GNCB of the Consiglio Nazionale delle Ricerche.

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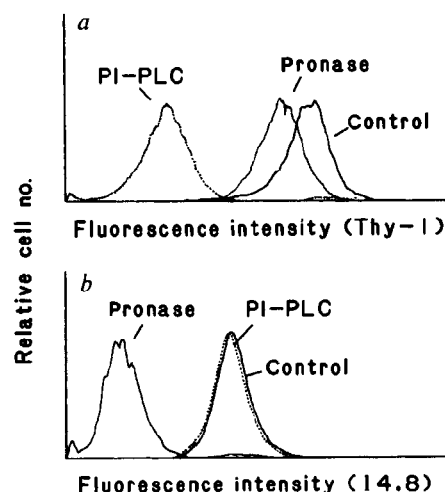
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Errata

Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein

M. G. Low & P. W. Kincade
Nature **318**, 62-64 (1985)

IN Fig. 1a and b, two lines representing the incubation with phosphatidylinositol-specific phospholipase C (PI-PLC) were not properly reproduced. The correct figure is shown here:



More help required on T and B cells

J. Shifflett
Nature **316**, 490 (1985)

THE word 'antigen' was omitted from the second sentence in paragraph 2, which should read: 'B lymphocytes might find it difficult to pinocytose a surface antigen of any pathogen unwilling to facilitate its own destruction...'. In addition, the second sentence in the third paragraph is made clearer with the words 'of materials' deleted.

Shanghai Institute of Biochemistry: The molecular biology revolution

A. Anderson
Nature **318**, 217-218 (1985)

IN this item in the 'Science in China' feature, the deputy-director of the Shanghai Institute of Biochemistry was inadvertently referred to as director. The director is Professor Lin Qi-shui, and his deputy is Professor Zhang You-shang.

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